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(54) Title: INHIBITOR OF STEM CELL PROLIFERATION AND USES THEREOF

(57) Abstract

Disclosed and claimed are methods for the isolation and use of stem cell inhibiting factors for regulating the abnormal stem cell cycle and for accelerating the post-chemotherapy peripheral blood cell recovery. Also disclosed and claimed are the inhibitors of stem cell proliferation.

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INHIBITOR OF STEM CELL PROLIFERATION AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to the use of inhibitors of stem cell proliferation for regulating stem cell cycle in the treatment of humans or animals having autoimmune diseases, aging, cancer, myelodysplasia, preleukemia, leukemia, psoriasis or other diseases involving hyperproliferative conditions. The present invention also relates to a method of treatment for humans or animals anticipating or having undergone exposure to chemotherapeutic agents, other agents which damage cycling stem cells, or radiation exposure. Finally, the present invention relates to the improvement of the stem cell maintenance or expansion cultures for auto and allo-transplantation procedures or for gene transfer.

BACKGROUND OF THE INVENTION

Most end-stage cells in renewing systems are short-lived and must be replaced continuously throughout life. For example, blood cells originate from a self-renewing population of multipotent hematopoietic stem cells (HSC). Because the hematopoietic stem cells are necessary for the development of all of the mature cells of the hematopoietic and immune systems, their survival is essential in order to reestablish a fully functional host defense system in subjects treated with chemotherapy or other agents.

Hematopoietic cell production is regulated by a series of factors that stimulate growth and differentiation of hematopoietic cells, some of which, for example erythropoietin and G-CSF, are currently used in clinical practice. One part of the control network which has not been extensively characterized, however, is the feedback mechanism that forms the negative arm of the regulatory process (Eaves *et al.* Blood 78:110-117, 1991).

Early studies by Lord and coworkers showed the existence of a soluble protein factor in normal murine and porcine bone marrow extracts, which was capable of reversibly inhibiting the cycling of HSC (Lord *et al.*, Br. J. Haem. 34:441-446, 1976). This inhibitory activity (50-100 kD molecular weight) was designated stem cell inhibitor (SCI).

Purification of this factor from primary sources was not accomplished due to the difficulties inherent in an *in vivo* assay requiring large numbers of irradiated mice. In an attempt to overcome these problems Pragnell and co-workers developed an *in vitro* assay for primitive hematopoietic cells (CFU-A) and screened cell lines as a source of the inhibitory activity.

As earlier studies had identified macrophages as possible sources for SCI (Lord *et al.* Blood Cells 6:581-593, 1980), a mouse macrophage cell line, J774.2, was selected (Graham *et al.* Nature 344:442-444, 1990). The conditioned medium from this cell line was used for purification; an inhibitory peptide was isolated which proved to be identical to the previously described cytokine macrophage inflammatory protein 1-alpha (MIP-1-alpha). Thus, MIP-1-alpha was isolated from a cell line, not from primary material. While Graham *et al.* observed that antibody to MIP-1-alpha abrogated the activity of a crude bone marrow extract, other workers have shown that other inhibitory activities are important. For example, Graham *et al.* (J. Exp. Med. 178:925-32, 1993) have suggested that TGFβ, not MIP-1α, is a primary inhibitor of hematopoietic stem cells. Further, Eaves *et al.* (PNAS 90:12015-19, 1993) have suggested that both MIP-1α and TGFβ are present at suboptimal levels in normal bone marrow and that inhibition requires a synergy between the two factors.

Other workers have described additional stem cell inhibitory factors. Frindel and coworkers have isolated a tetrapeptide from fetal calf marrow and liver extracts which has stem cell inhibitory activities (Lenfant *et al.*, PNAS 86:779-782, 1989). Paukovits *et al.* (Cancer Res. 50:328-332, 1990) have characterized a pentapeptide which, in its monomeric form, is an inhibitor and, in its dimeric form, is a stimulator of stem cell cycling. Other factors have also been claimed to be inhibitory in various *in vitro* systems (cf. Wright and Pragnell in Bailliere's Clinical Haematology v. 5, pp. 723-39, 1992 (Bailliere Tinadall, Paris)).

To date, none of these factors have been approved for clinical use. However, the need exists for effective stem cell inhibitors. The major toxicity associated with chemotherapy or radiation treatment is the destruction of normal proliferating cells which can result in bone

marrow suppression or gastrointestinal toxicity. An effective stem cell inhibitor would protect these cells and allow for the optimization of these therapeutic regimens. Just as there is a proven need for a variety of stimulatory cytokines (e.g., G-CSF, GM-CSF, erythropoietin, IL-11) depending upon the clinical situation, so too it is likely that a variety of inhibitory factors will be needed to address divergent clinical needs.

I. Chemotherapy and Radiotherapy of Cancer

Productive research on stimulatory growth factors has resulted in the clinical use of a number of these factors (erythropoietin, G-CSF, GM-CSF, etc.). These factors have reduced the mortality and morbidity associated with chemotherapeutic and radiation treatments. Further clinical benefits to patients who are undergoing chemotherapy or radiation could be realized by an alternative strategy of blocking entrance of stem cells into cell cycle thereby protecting them from toxic side effects.

II. Bone Marrow Transplantation

Bone marrow transplantation (BMT) is a useful treatment for a variety of hematological, autoimmune and malignant diseases. *Ex vivo* manipulation of cells is currently being used to expand primitive stem cells to a population suitable for transplantation. Optimization of this procedure requires: (1) sufficient numbers of stem cells able to maintain long term reconstitution of hematopoiesis; (2) the depletion of graft versus host-inducing T-lymphocytes and (3) the absence of residual malignant cells. This procedure can be optimized by including a stem cell inhibitor(s) for *ex vivo* expansion.

The effectiveness of purging of bone marrow cells with cytotoxic drugs in order to eliminate residual malignant cells is limited due to the toxicity of these compounds for normal hematopoietic cells and especially stem cells. There is a need for effective protection of normal cells during purging; protection can be afforded by taking stem cells out of cycle with an effective inhibitor.

III. Peripheral Stem Cell Harvesting

Peripheral blood stem cells (PBSC) offer a number of potential advantages over bone marrow for autologous transplantation. Patients without suitable marrow harvest sites due to tumor involvement or previous radiotherapy can still undergo PBSC collections. The use of blood stem cells eliminates the need for general anesthesia and a surgical procedure in patients who would not tolerate this well. The apheresis technology necessary to collect blood cells is efficient and widely available at most major medical centers. The major limitations of the method are both the low normal steady state frequency of stem cells in peripheral blood and their high cycle status after mobilization procedures with drugs or growth factors (e.g., cyclophosphamide, G-CSF, stem cell factor). An effective stem cell inhibitor would be useful to return such cells to a quiescent state, thereby preventing their loss through differentiation.

IV. Treatment of Hyperproliferative Disorders

A number of diseases are characterized by a hyperproliferative state in which dysregulated stem cells give rise to an overproduction of end stage cells. Such disease states include, but are not restricted to, psoriasis, in which there is an overproduction of epidermal cells, and premalignant conditions in the gastrointestinal tract characterized by the appearance of intestinal polyps. A stem cell inhibitor would be useful in the treatment of such conditions.

V. Gene Transfer

The ability to transfer genetic information into hematopoietic cells is currently being utilized in clinical settings. The bone marrow is a useful target for gene therapy because of ease of access, extensive experience in manipulating and treating this tissue *ex vivo* and because of the ability of blood cells to permeate tissues. Furthermore, the correction of certain human genetic defects may be possible by the insertion of a functional gene into the primitive bone marrow stem cells of the human hematopoietic system.

There are several limitations for the introduction of genes into human hematopoietic

cells using either retrovirus vector or physical techniques of gene transfer: (1) The low frequency of stem cells in hematopoietic tissues has necessitated the development of high efficiency gene transfer techniques; and (2) more rapidly cycling stem cells proved to be more susceptible to vector infection, but the increase of the infection frequency by stimulation of stem cell proliferation with the growth factors is shown to produce negative effect on long term gene expression,because cells containing the transgenes are forced to differentiate irreversibly and loose their self-renewal. These problems can be ameliorated by the use of a stem cell inhibitor to prevent differentiation and loss of self-renewal.

SUMMARY OF THE INVENTION

The present invention is an inhibitor of stem cell proliferation characterized by the following properties:

(a) Specific activity (IC_{50}) less than or equal to 20 ng/ml in a murine colony-forming spleen (CFU-S) assay (see example 4)

(b) Molecular weight greater than 10,000 and less than 100,000 daltons (by ultrafiltration)

(c) Activity sensitive to degradation by trypsin

(d) More hydrophobic than MIP-1α or TGFβ and separable from both by reverse phase chromatography (cf. Example 12)

(e) Biological activity retained after heating for one hour at 37°C, 55°C or 75°C in aqueous solution

(f) Biological activity retained after precipitation with 1% hydrochloric acid in acetone

The present invention is further characterized and distinguished from other candidate stem cell inhibitors (e.g., MIP-1α, TGFβ and various oligopeptides) by its capacity to achieve inhibition in an *in vitro* assay after a short preincubation period (see Example 5).

The present invention also comprises pharmaceutical compositions containing INPROL for treatment of a variety of disorders.

The present invention provides a method of treating a subject anticipating exposure to an agent capable of killing or damaging stem cells by administering to that subject an effective amount of a stem cell inhibitory composition. The stem cells protected by this method may be hematopoietic stem cells ordinarily present and dividing in the bone marrow. Alternatively, stem cells may be epithelial, located for example, in the intestines or scalp or other areas of the body or germ cells located in reproductive organs. The method of this invention may be desirably employed on humans, although animal treatment is also encompassed by this method.

In another aspect, the invention provides a method for protecting and restoring the hematopoietic, immune or other stem cell systems of a patient undergoing chemotherapy, which includes administering to the patient an effective amount of INPROL.

In still a further aspect, the present invention involves a method for adjunctively treating any cancer, including those characterized by solid tumors, by administering to a patient having cancer an effective amount of INPROL to protect stem cells of the bone marrow, gastrointestinal tract or other organs from the toxic effects of chemotherapy or radiation therapy.

Yet another aspect of the present invention involves the treatment of leukemia comprising treating bone marrow cells having proliferating leukemia cells therein with an effective amount of INPROL to inhibit proliferation of normal stem cells, and treating the bone marrow with a cytotoxic agent to destroy leukemia cells. This method may be enhanced by the follow-up treatment of the bone marrow with other agents that stimulate its proliferation; e.g., colony stimulating factors. In one embodiment this method is performed *in vivo*. Alternatively, this method is also useful for *ex vivo* purging and expansion of bone marrow cells for transplantation.

In still a further aspect, the method involves treating a subject having any disorder caused by proliferating stem cells. Such disorders, such as psoriasis, myelodysplasia, some autoimmune diseases, immuno-depression in aging, are treated by administering to the subject an effective amount of INPROL to partially inhibit proliferation of the stem cell in question.

The present invention provides a method for reversibly protecting stem cells from damage from a cytotoxic agent capable of killing or damaging stem cells. The method involves administering to a subject anticipating exposure to such an agent an effective amount of INPROL.

The present invention includes within its scope the purification of porcine INPROL and anticipates the use of such purified protein to obtain sequence data useful for cloning the cDNA or the gene for INPROL.

The present invention also provides:

An inhibitor of stem cell proliferation isolated from porcine or other bone marrow by the following procedure (cf. Example 12):

(a) Extraction of bone marrow and removal of particulate matter through filtration

(b) Heat treatment at 56°C for 40 minutes followed by cooling in ice bath

(c) Removal of precipitate by centrifugation at 10,000 g for 30 minutes at 4°C

(d) Acid precipitation by addition of supernatant to 10 volumes of stirred ice-cold acetone containing 1% by volume concentrated hydrochloric acid and incubation at 4°C for 16 hours

(e) Isolation of precipitate by centrifugation at 20,000 g for 30 minutes at 4°C and washing with cold acetone followed by drying

(f) Isolation by reverse phase chromatography and monitoring activity by inhibition of colony formation by bone marrow cells pretreated with 5-fluorouracil and incubated in the presence of murine IL-3, as well as by absorption at 280 nm and by SDS-PAGE

The present invention also provides:

A method for purifying an inhibitor of stem cell proliferation substantially free from other proteinaceous materials comprising the preceding steps, as also described in more detail below.

The present invention also provides:

A method of treatment for humans or animals wherein an inhibitor of stem cell proliferation functions to ameliorate immunosuppression caused by stem cell hyperproliferation.

The present invention also provides:

A method of treatment for humans or animals wherein said inhibitor of stem cell proliferation is administered after the stem cells are induced to proliferate by exposure to a cytotoxic drug or irradiation procedure. Stem cells are normally quiescent but are stimulated to enter cell cycle after chemotherapy. This renders them more sensitive to a second administration of chemotherapy; the current method protects them from this treatment.

The present invention also provides:

A method of treatment for humans or animals wherein said inhibitor of stem cell proliferation is administered as an adjuvant before or together with vaccination for the purpose of increasing immune response.

The present invention also provides:

A method of treatment for humans or animals receiving cytotoxic drugs or radiation treatment which comprises administering an effective amount of the inhibitor of stem cell proliferation to protect stem cells against damage.

The current invention describes an inhibitor of stem cells, termed INPROL, which is different from those known in the art such as MIP-1-alpha, TGF-beta, the tetrapeptide of Frindel and colleagues or the pentapeptide of Paukovits and coworkers (cf., Wright & Pragnell, 1992 (*op cit*)). INPROL has a molecular weight exceeding 10,000 daltons by ultrafiltration which distinguishes it from the tetrapeptide as well as the pentapeptide. It is more hydrophobic than MIP-1 alpha or TGF beta in reverse phase chromatography systems, distinguishing it from those cytokines. Further, its mode of action is different from that of any previously described inhibitor in that it is active in an *in vitro* assay when used during a preincubation period only. MIP-1-alpha for example, is not effective when used during a pre-incubation period only (Example 5). Further, INPROL is active in an assay measuring "high

proliferative potential cells" (HPP-PFC) whereas MIP-1-alpha is not (Example 6).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1-4 show an SDS polyacrylamide gel run of the product after each stage of purification.

Figure 5 shows an HPLC chromatogram of the final purification.

Figure 6 shows tritiated thymidine incorporation (cpm) into cells of the FDCP-mix line without (Control = 100%) and with various concentrations of INPROL. Data are normalized against the control value.

Figure 7 shows the percent of cells in the S phase of the cell cycle after treatment of mice with testosterone propionate (TSP), TSP plus INPROL, or vehicle (Control). Each group contained 25 animals (3-4 per time point).

Figure 8 shows survival of mice treated with two doses of 5-FU, with or without INPROL treatment. Each group contained 30 animals.

Figure 9 shows survival of irradiated mice, with and without INPROL treatment. Each group contained 50 animals.

Figures 10 A and 10 B show regeneration of normal bone marrow long term culture cells 1 week (10 A) and 3 weeks (10 B) after treatment with Ara-C or Ara-C plus INPROL.

Figure 11 shows survival of mice (75 per group) after lethal irradiation and transplantation of $3x10^4$ bone marrow cells after pre-incubation with medium (Control) or INPROL (25 ng/ml) for 4 hours. Survival was monitored for 30 days.

Figure 12 shows CFU-GM number formed after 14 days in culture by bone marrow cells from mice after lethal irradiation and restoration with donor bone marrow cells pre-incubated with INPROL or medium for 4 hours.

Figure 13 shows suspension cells from lymphoid long-term culture which were taken every week, washed out, and plated with IL-7 (10 ng/ml) after preincubation with medium or INPROL for 4 hours.

Figure 14 shows improved repopulating ability of leukemic peripheral blood cells treated with INPROL. Long term culture initiating cells (LTC-IC) were measured by plating adherent and nonadherent LTC cells with and without INPROL, and scoring CFU-GM on day 7. Data are normalized to control values.

Figure 15A shows a C4 reverse phase chromatogram of purified INPROL eluting at 53% acetonitrile. Figure 15B shows a C4 reverse phase chromatogram of MIP-1 alpha eluting at 43.9% acetonitrile. Figure 15C shows an SDS-PAGE chromatogram of the crude INPROL preparation and of the purified preparation after reverse phase.

In order that the invention herein described may be more fully understood, the following detailed description is set forth. This description, while exemplary of the present invention, is not to be construed as specifically limiting the invention and such variations which would be within the purview of one skilled in this art are to be considered to fall within the scope of this invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

INPROL reversibly inhibits division of stem cells. Specifically, INPROL is effective in temporarily inhibiting cell division of hematopoietic stem cells. Thus, the method of this invention may be employed in alleviating the undesirable side effects of chemotherapy on the patient's hematopoietic, myeloid and immune systems by protecting stem cells from damage caused by chemotherapeutic agents or radiation used to destroy cancer or virally infected cells. In one embodiment of the invention, INPROL is administered to the patient in a dosage sufficient to inhibit stem cell division while the chemotherapeutic agent acts on diseased cells. After the chemotherapeutic agent has performed its function, the stem cells inhibited by INPROL will, without further treatment, revert to dividing cells. If it is desired to enhance the regeneration of hematopoiesis, stimulatory growth factors or cytokines may be used in addition.

In a typical clinical situation, INPROL is administered to a patient by intravenous

injection or infusion using, for example, 0.01 to 1 mg/kg of purified or recombinant INPROL administered, e.g., 4-60 hours prior to standard chemotherapy or radiation treatments. In another embodiment of the invention, pretreatment with INPROL allows for increased doses of chemotherapeutic agents or of radiation beyond doses normally tolerated in patients. Optionally, stimulatory growth factors such as G-CSF, stem cell factor, are used after chemotherapy or radiation treatment to further improve hematopoietic reconstitution.

In another embodiment of the invention, INPROL is employed in a method for preparing autologous bone marrow for transplantation. The marrow is treated *ex vivo* with an effective amount of INPROL to inhibit stem cell division and then purged of cancerous cells by administering to the marrow cultures an effective amount of a chemotherapeutic agent or radiation. Chemotherapy agents with specificity for cycling cells are preferred. Marrow thus treated is reinjected into the autologous donor. Optionally, the patient is treated with an agent known to stimulate hematopoiesis to improve hematopoietic reconstitution of the patient.

In another embodiment of the invention, INPROL is employed as an adjunctive therapy in the treatment of leukemia. For example, in disease states where the leukemic cells do not respond to INPROL, the leukemic bone marrow cells are treated *ex vivo*, with INPROL. The proliferation of normal stem cells is prevented by administration of INPROL. Thus, during the time that the proliferating leukemic cells are treated with a cell cycle-specific cytotoxic agent, a population of normal stem cells is protected from damage. Additionally, a stimulatory cytokine, such as IL-3 or GM-CSF, is optionally administered to induce cycling in the leukemic cells during drug or radiation treatment while the normal stem cells are protected with INPROL. The patient is treated with chemotherapy agents or radiation to destroy leukemic cells, and the purged marrow is then transplanted back into the patient to establish hematopoietic reconstitution.

Similarly, in another embodiment of the invention for treatment of patients with serious viral infections that involve blood cells or lymphocytes, such as HIV infection, bone marrow is treated *ex vivo* with INPROL followed by antiviral agents, drugs which destroy infected cells, or antibody-based systems for removing infected cells. Following myeloablative antiviral or

myeloablative chemotherapy to eradicate viral host cells from the patient, the INPROL-treated marrow cells are returned to the patient.

In another embodiment of the invention, INPROL is employed to treat disorders related to hyperproliferative stem cells. For example, psoriasis is a disorder caused by hyperproliferating epithelial cells of the skin and is sometimes treated with cytotoxic drugs. Other pre-neoplastic lesions in which stem cell proliferation is involved are also amenable to effective amounts of INPROL employed to inhibit wholly or partially the proliferation of the stem cells. For these uses, topical or transdermal delivery compositions containing INPROL are employed where appropriate, as an alternative to parenteral administration. In most cases of leukemia, the leukemia progenitors are differentiated cell populations which are not affected by INPROL and which are therefore treated by methods using INPROL such as those described above. In cases where leukemia progenitors are very primitive and are directly sensitive to inhibition by INPROL, proliferation of leukemia cells is attenuated by administration of effective amounts of INPROL.

Antibodies, monoclonal or polyclonal, are developed by standard techniques to the INPROL polypeptides. These antibodies or INPROL polypeptides are labelled with detectable labels of which many types are known in the art. The labelled INPROL or anti-INPROL antibodies are then employed as stem cell markers to identify and isolate stem cells by administering them to a patient directly for diagnostic purposes. Alternatively, these labelled polypeptides or antibodies are employed *ex vivo* to identify stem cells in a bone marrow preparation to enable their removal prior to purging neoplastic cells in the marrow. In a similar manner, such labelled polypeptides or antibodies are employed to isolate and identify epithelial or other stem cells. In addition, such antibodies, labelled or unlabelled, are used therapeutically through neutralization of INPROL activity or diagnostically through detection of circulating INPROL levels.

The porcine factor is employed in the methods of this invention, provided that it provokes no significant deleterious antibody generation from the human immune system. An analogous or homologous human protein with activity similar to that of INPROL is within the

scope of the invention. Such a protein is cloned from human gene or cDNA libraries for expression of recombinant human INPROL using standard techniques. For example, using sequence information obtained from the purified protein, oligonucleotide probes are constructed which can be labelled, e.g., with 32-phosphorus and used to screen an appropriate cDNA library (e.g., from bone marrow). Alternatively, an expression library from an appropriate source (e.g., bone marrow) is screened for cDNA's coding for INPROL using antibody or using an appropriate functional assay (e.g., that described in Example 2).

Homologous or analogous versions of INPROL from other species are employed in various veterinary uses, similar to the therapeutic embodiments of the invention described above.

Further, the stem cell inhibitory factor acts on cycling stem cells by reversibly placing them in an undividing "resting" state. When it is desirable to stimulate the quiescent stem cells into division, e.g., after treatment of a patient with cancer chemotherapy agents or radiation, colony-stimulating factors and other hematopoietic stimulants are administered to the subject. Examples of such factors include but are not limited to: M-CSF, CSF-1, GM-CSF, G-CSF, Megakaryocyte-CSF or other cytokines, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-14, or erythropoietin .

INPROL polypeptides or active fragments having stem cell inhibitory activity are purified or synthesized by conventional chemical processes combined with appropriate bioassays for stem cell inhibitory activity, as exemplified in the protocols described below.

In one embodiment of the invention, a therapeutically effective amount of the INPROL protein or a therapeutically effective fragment thereof is employed in admixture with a pharmaceutically acceptable carrier. This INPROL composition is generally administered by parenteral injection or infusion. Subcutaneous, intravenous, or intramuscular injection routes are selected according to therapeutic effect achieved.

When systematically administered, the therapeutic composition for use in this invention is in the form of a pyrogen-free, parenterally acceptable aqueous solution. Pharmaceutically acceptable protein solution, having due regard to pH, isotonicity, stability, carrier proteins and

the like, is within the skill of the art. For administration in the method for treating hyperproliferating stem cells, the composition containing INPROL is administered topically or through a transdermal patch to localize and optimize its effect on the area of hyperproliferation.

The dosage regimen involved in a method for treating the subject anticipating exposure to such cytotoxic agents or for treatment of hyperproliferating stem cells is determined by the attending physician considering various factors which modify the action of drugs; e.g., the condition, body weight, sex, and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, the daily regimen is in the range of 1-100 μg/kg micrograms of INPROL protein or fragment thereof per kilogram of body weight.

Following the subject's exposure to the cytotoxic agent or radiation, the therapeutic method of the present invention optionally employs administering to the subject one or more lymphokines, colony stimulating factors or other cytokines, hematopoietins, interleukins, or growth factors to generally stimulate the growth and division of the stem cells (and their descendants) inhibited by the prior treatment with INPROL. Such therapeutic agents which encourage hematopoiesis include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, Meg-CSF, M-CSF, CSF-1, GM-CSF, G-CSF or erythropoietin. The dosages of these agents are selected according to knowledge obtained in their use in clinical trials for efficacy in promoting hematopoietic reconstitution after chemotherapy or bone marrow transplant. These dosages would be adjusted to compensate for variations in the physical condition of the patient, and the amount and type of chemotherapeutic agent or radiation to which the subject was exposed. Progress of the reversal of the inhibition of the stem cells caused by administration of INPROL in the treated patient is monitored by conventional methods.

In the treatment of leukemia, it is beneficial to administer both INPROL to inhibit normal stem cell cycling and a stimulator of leukemic cell growth, such as IL-3 or GM-CSF, simultaneously with the cytotoxic drug treatment or during irradiation. By this protocol, it is possible to achieve the greatest differences between the cycling statuses and drug sensitivities of normal and leukemic cells.

Example 1: *In Vivo* Stem Cell Proliferation Inhibition Assay

For the detection of stem cells proliferation the number of CFUs in S-phase of cell cycle was measured by ^{3}H-Thymidine "suicide" method (Becker *et al.*, Blood 26:296-308,1965).

Immature hematopoietic progenitors--Colony Forming Units in spleen (CFU-S)-- can be detected *in vivo* by forming macroscopic colonies in the spleens of lethally irradiated mice, 8-12 days after the intravenous injection of hematopoietic cells (Till & McCulloch, 1961).

For the standard CFUs proliferation assay the method of H-Thymidine "suicide" is usually applied (Becker *et al.*, 1965). The method is based on incorporation of radiolabelled Thymidine (^{3}H-Thymidine) a precursor of DNA into cells during DNA synthesis. The CFU-S which are in S-phase of cycle at the time of testing, are killed by the high radioactivity and therefore not able to form colonies in spleen. Thus, the difference between the number of CFU-S formed by the injection of the cell sample incubated without ^{3}H-Thymidine and the same cells incubated with ^{3}H-Thymidine shows the percentage of the proliferating CFU-S in the original sample.

The inhibitor testing can not be done with the bone marrow stem cell population from unstimulated animals, as far as the inhibitor does effect on cycling CFUs, which are as low as 7-10% in the bone marrow of normal mice.

To stimulate CFUs proliferation phenylhydrazine (PHZ) stimulation, or sublethal irradiation were used (Lord, 1976).

We have invented the injection of testosterone-propionate (TSP) based on its stimulatory effect on CFUs cycling (Byron *et al.*, Nature 228:1204, 1970) which simplified the testing and did not cause any side effects. The TSP induced stimulation of CFUs proliferation within 20-24 hours after injection and the effect could be seen at least 7 days.

The procedure used for the screening of the fractions during purification of the Inhibitor was as follows:

Mice: BDF_1 or CBF_1 mice strains were used throughout all testing.

Donor mice were treated with TSP in dose 10 mg/100 g by intraperitoneal injection of 0.2 ml/mouse in order to induce 30-35% CFUs in S-phase.

Twenty-four hours later the bone marrow is to be taken from the femurs for the cell suspension preparation. Five to ten million cells per ml are then incubated with different control and test fractions for 3.5 hours at 37°C in water bath, with two tubes for each group (hot and cold now).

After 3.5 hours, ^{3}H-Thymidine (1 mCi/ml, specific activity 18-25 Ci/mmole) is to be added to each hot tube in volume 200 µl per 1 ml of cell suspension, nothing add to the cold tubes, continue incubation 30 more minutes at 37°C.

After 30 minute incubation, the kill reaction is to be terminated by adding 10 ml of cold (4°C) medium containing 400 ug/ml nonradioactive Thymidine. Cells are washed off extensively (3 times).

Cells are to be resuspend and diluted to a desirable concentration for the injections usually 2-4 x 10^4 cells per mouse in 0.3-0.5 ml.

Recipient mice 8-10 per group are to be irradiated not later than 6 hours before the injections.

Harvest recipients spleens on day 9-12 and fix in Tellesnitsky's solution, score colonies by eye. Calculate the percentage of cells in S-phase using the formula.

where a -- CFUs number with ^{3}H-Thymidine

where b -- CFUs number with ^{3}H-Thymidine

The test data of INPROL presented in Table 4 demonstrated that cycling stem cells after treatment with INPROL become resistant to the action of ^{3}H-Thymidine. The same is true for the S-phase specific cytotoxic drug cytosine arabinoside and hydroxyurea (data not shown). If the treated stem cells are then washed with the cold media containing non-radioac' Thymidine the surviving stem cells proliferate in mice spleens to form colonies normal'

Table 4

Inhibitory Activity Of INPROL On CFUs Proliferation
During Four Hour Incubation With Bone Marrow Cells

Group	CFUs Per 2 X -^{3}H-TdR	10^4 Cells + ^{3}H-TdR	Percent CFUs Killed by ^{3}H-TdR
No incubation	22.2 ± 2.0	13.7 ± 2.4	38.3 ± 1.7
4 Hour with Media	18.7 ± 3.0	11.4 ± 1.3	43.1 ± 1.4
4 Hour with INPROL	21.2 ± 2.3	20.7 ± 2.6	2.1 ± 0.08

Example 2: *In Vitro* Stem Cell Proliferation Inhibition Assay

Using the following test system (Lord *et al.*, in The Inhibitors of Hematopoiesis pp. 227-239, 1987) the direct effect of INPROL was shown. The multilineage factor (IL-3) dependent stem cell line, FDCP mix A4 (A4) were maintained in IMDM medium supplemented with 20% horse serum and 10% WEHI-3-conditioned medium as a source of colony-stimulating IL-3.

Tritiated Thymidine incorporation assay for the proliferation measured: A4 cells 5 x 10^4 in 100 µl medium with 20% horse serum and 50% of WEHI-3 CM were incubated at 37°C in 5% CO^2 for 16 hours.

INPROL or the crude BME (fraction IV) were added at the start. 3HtdR (3.7KBq in 50 µl at 740 GBq/mmole) was then added to each group for a further 3 hours of incubation. The level of proliferation was measured by harvesting cells.

$$\% \text{ Inhibition} = \frac{\text{cpm without INPROL - cpm with INPROL}}{\text{cpm without INPROL}} \times 100\%$$

Incorporation of tritiated thymidine (3H-Tdr) by FDCPmix-A4 cells grown in presence of graded doses of normal bone marrow extract or INPROL is depicted on Figure 6. It can be seen that purified composition of INPROL is at least 1,000 times more active than the starting

material. Time of exposure (16 hours) is an important factor for effective inhibition and shows the evidence of the direct effect on stem cells of A4 cell line.

Example 3: Inhibition of CFUs Proliferation by INPROL Injected *in vivo*: Doses and the Duration of the Effect

The studies of the effect of INPROL injected *in vivo* revealed that INPROL can effectively block the recruitment of CFUs into cycle, thus protecting those cells from the cytotoxic effect of further treatment showing its potential for clinical use.

The experimental protocol had two goals: to check effect of INPROL on CFUs when injected *in vivo* and to define the effective duration of INPROL activity in relation to cycling stem cells.

To stimulate CFUs proliferation, the injection of testosterone-propionate was used based on the effect mentioned above in Example 1.

Mice BDF1 were injected with TSP (10 mg/100 g) on Day 0; 24 hours later mice of each experimental group (4 mice per group) received a single SCPI injection at dose range 0 μg, 5 μg, 10 μg, 15 μg/mouse i.p.

Twenty-four hours after SCPI injection, mice were sacrificed and the percent of cycling CFU-S was measured by the assay described in Example 1. TSP injection induced about 50% CFU-S into cycling in comparison with 7% in untreated mice. INPROL in doses as low as 2 μg/mouse was able to inhibit TSP induced proliferation down to the normal level.

For the duration of the effect evaluation, one group of mice (21 mice per group) was injected with TSP only and another group was injected both with TSP and INPROL (24 hours after TSP). The CFU-S cycling was measured every 24 hours during a week by taking 3 donors from each group and measuring CFU-S cycle status in their bone marrow by method described (see Example 1). Data presented in Figure 7 show that while the duration of the effect of TSP is at least 7 days, a single injection of INPROL can place CFU-S into quiescence and keep them out of cycle for no more than 48-72 hours. Since the majority of chemotherapeutic agents used for cancer and leukemia chemotherapy have a relatively short *in*

vivo half-life, usually less than 24 hours, the INPROL effect according to the data obtained is maintained for longer than the effective time during which the chemotherapeutic agents like cytosine arabinoside or hydroxyurea are active *in vivo*. More important, chemotherapeutic and radiation treatment having longer intervals (more than 24 hours and less than 96 hours) are needed between the first (non-damaging for the stem cells) and the second, damaging CFUs cytotoxic procedure, the single injection of INPROL during the intervals between two applications of chemotherapeutic agent or radiation should be sufficient. For several repeatable cycles of cytotoxic therapy or radiation the same strategy could be applied based on duration of INPROL effect.

Example 4: Most Primitive Hematopoietic Stem Cells Stimulated to Cycle Rapidly After Treatment with 5-FU are Protected by INPROL from the Second 5-FU Exposure

The drug 5-fluorouracil (5-FU) drastically reduces the number of cells in the myeloid and lymphoid compartments. It is usually thought of as being cell-cycle specific, targeting rapidly proliferating cells because incorporation of the nucleotide analogue into DNA during S-phase of the cell cycle or before results in cell death. The long-term survival and immunohematopoietic reconstitution of the bone marrow of mice is not affected by a single dose of 5-FU; however, it was demonstrated (Harrison *et al.* Blood 78:1237-1240, 1991) that pluripotent hematopoietic stem cells (PHSC) become vulnerable to a second dose of 5-FU for a brief period about 3-5 days after the initial dose. It can be explained that PHSC normally cycle too slowly for a single dose of 5-FU to be effective and are stimulated into rapid cycling by stimuli resulting from the initial 5-FU treatment. We have proposed that PHSC can be returned to a slow cycle status by INPROL and thus protected from the second 5-FU treatment.

The mice used in these experiments were BDF1 male mice. A stock solution of 5-FU (Sigma) was prepared in physiologic saline at a concentration of 10 μg/mL. Each treated mouse received 2 mg of 5-FU per 10 g body weight via a tail vein at Day 0 of the experiment;

24 hours later mice were injected with INPROL (10 μg/100 g of body weight) intraperitoneally and on Day 3 were injected with the second dose of 5-FU. The survival study was conducted by monitoring the death of mice in experimental (treatment with INPROL) and control groups of 30 mice each. The survival curves are shown in Figure 8.

Example 5: Effects of Pre-Incubation with INPROL vs. MIP 1 Alpha in Bone Marrow Cells

The purpose of this experiment was to compare the inhibitory effects of pre-incubation with Inprol and MIP 1 alpha on mouse bone marrow cells *in vitro*.

The following procedure was used:

in vivo: BDF1 mice, 6-15 weeks of age, are injected with 200 mg/kg 5FU i.p. 48 hours before harvesting marrow from the femurs.

in vitro: A single cell pooled suspension is counted and 5 x 10^6 cells are incubated in a total of 2 mls with or without Inprol or MIP-1 alpha, 5% horse serum, IMDM media with added L-glutamine, at 37°C and 5% CO_2 for 4 hours. The cells are then washed twice and recounted. They are plated in methylcellulose in the following final conditions:

0.8% methylcellulose

25% horse serum

20 ng/ml recombinant murine IL3

L-glutamine added

5x 10^5 cells per ml

IMDM media

Plates were incubated for 11 days at 37°C and 5% CO_2 in 100% humidity. Colonies more than 50 cells were counted.

Groups	Colony Number	Percent of Control
Control	31.0	100%
Inprol	21.25	68.5%
MIP 1 alpha	35.25	114%

Example 6: INPROL inhibits HPP-CFC proliferation

An *in vitro* assay for assessing murine reconstituting stem cells and early precursors is the high proliferative potential colony (HPP-PFC) assay; other related assays, e.g., CFU-A, CFU-GM, CFU-E, and CFU-GEMM, detect progressively restricted progenitor populations (M. Moore, Blood 177:2122-2128, 1991). This example shows that pretreatment of cells with INPROL inhibits their proliferation, whereas MIP-1α fails to do so under these experimental conditions.

BDF1 mice were treated with 5-fluorouracil (200 mg/kg i.p.) before their bone marrow was assayed for HPP-CFC numbers. Cells were washed by centrifugation and incubated at densities of 10^6 to 5x10^6 /ml in medium with either no added agent (Controls), INPROL (25 ng/ml) or MIP-1α (200 ng/ml) for 4 hours. After incubation, cells were washed and plated in agar (0.3%) with 30% FCS and combined conditioned medium from 5637 and WEHI-3B cell

lines (7.5% of each conditioned medium, as recommended by Sharp et al., 1991). Plating concentration was $5x10^4$ cells/ml in 60 mm dishes. Colonies were scored on day 14 and the results are indicated below.

Group	HPP-CFU	% of Control
Control	15.5±1.2	100 %
INPROL	8.3±0.7	53.5 %
MIP-1	15.8±0.9	101%

According to these results, MIP-1α did not inhibit proliferation of the most immature precursors when present only during the pre-incubation period. INPROL did effectively inhibit proliferation under these conditions, indicating fundamental differences between INPROL and MIP-1α in terms of biological activity.

Example 7: INPROL Therapy Effect on the Recovery from Radiation-induced Bone Marrow Aplasia

Bone marrow aplasia is the primary limiting toxicity of radiation cancer therapy. It has been demonstrated that some growth factors (e.g., G-CSF, GM-CSF, erythropoietin) can accelerate recovery from radiation-induced bone marrow aplasia. The concept of protection by using an inhibitor of stem cell proliferation is a different and complementary approach in coping with hematological damage. To follow the treatment procedure developed earlier (Examples 3, 4) a model of lethal irradiation of mice was established. It is known in the art that mice receiving 9Gy of cobalt 60 start dying after 10-14 days; by Day 30, mortality approximates 50%. This lethal dose was used in our model by splitting it into two subsequent applications of 4.5Gy each with an interval 3 days between doses. Preliminary data showed that the survival curve in that model was very close to that known for a single irradiation with 9Gy; moreover the test for the CFUs proliferation showed that 24 hours after the first

irradiation, 35-50% of CFUs are induced to proliferate. Such cells may be protected by a stem cell inhibitor delivered prior to the second dose.

To examine this possibility, mice (50 mice/group) received 4.5Gy on Day 0, INPROL 2 μg/mouse i.p. 24 hours later, control group was injected with saline, and the second dose of radiation (4.5 Gy) was given on Day 3.

Fig. 9 shows the increased survival after a single dose of INPROL. The conditions of the model are clinically relevant for treating any cancer, including those characterized by solid tumors, such treatment would be administered to a patient having cancer by delivering an effective dose of INPROL between two consecutive dosages of radiation, thereby allowing greater dosages of radiation to be employed for treatment of the cancer. It should also be possible to extend this modality to chemotherapeutic agents.

Example 8: INPROL Use for the Autologous Bone Marrow Transplantation

Bone marrow transplantation is the only known curative therapy for several leukemias (CML, AML, and others). *Ex vivo* conditioning of autologous BMT for infusion should provide the potential autologous sources of normal stem cells free of leukemic contamination and able to repopulate the recipients hematopoietic system to allow aggressive and effective therapy.

1. Long-term Bone Marrow Culture L1210 Leukemia Model For The Study Of INPROL Effect Preserving Normal Hematopoiesis During Purging With AraC.

Long-Term Bone Marrow Cultures (LTBMC) were established according to Toksoz *et al.* (Blood 55:931-936, 1980) and leukemic cell line L1210 was adopted to the LTBMC by cocultivation during 2 weeks. The simultaneous growth of normal and leukemic progenitors occurred in these combined LTBMC/L1210 cultures, similar to the situation in the bone marrow of a leukemic patient. Discrimination between normal colony forming units CFU and leukemic CFU was possible by growing them as agar colonies in the presence or absence of the conditioned medium from WEHI-3 (a murine IL-3 producing cell line). Normal cells

undergo apoptosis in the absence of IL-3 whereas leukemic cells can form colonies in its absence. Suspension cells from LTBMC-L1210 composition give approximately 150 colonies in presence of IL-3 (normal hematopoietic clones) and 70 colonies when growing without IL-3 (leukemic clones) per 50,000 cells plated.

The procedure of purging was as follows: on Day 0 all suspension cells and media (10 ml/flask) were taken off the flasks with LTBMC-L1210 and replace with 2 ml of media containing 200 µg cytosine arabinoside (AraC) (Tsyrlova *et al.* in Leukemia: Advances in Biology and Therapy v. 35, 1988); after 20 hours of incubation, flasks were washed out and replaced with 2 ml of fresh media alone (control group) or media containing INPROL at 25 ng/ml for 4 hours. After this preincubation cells were incubated again with 100 ug/flask AraC for 3 hours at 37°C. Each group contained 4 flasks. LTBMC-L1210 cultures were washed 3 times and replaced with fresh LTBC media; they were maintained as before for the regeneration studies for 3-4 weeks.

Data are presented in Fig. 10. There was no cell growth seen in control cultures treated with AraC only, while in INPROL protected flasks regeneration of hematopoiesis occurred much more rapidly due to proliferation of progenitors from the adherent layer. Moreover, the cells from the experimental group when plated in agar grew only in the presence of IL-3 giving about 100 CFU per 50,000 cells; no leukemic cell growth was observed at least during 4 weeks. Thus, marrow treated *ex vivo* with an effective dose of AraC in combination with INPROL can be purged of cancerous cells while the stem cells are be protected. It should be possible to extend this modality to other forms of chemotherapy or radiation treatments.

2. Marrow Repopulating Ability (MRA) And Thirty Days Radioprotection Are Increased By INPROL Treatment *In Vitro*.

MRA, the ability of cells to repopulate the bone marrow of lethally irradiated mice, together with the ability to confer radioprotection for 30 days is a direct *in vivo* measurement of the potential to rescue myelosuppressed animals (Visser *et al.* Blood Cells 14:369-384, 1988).

For radioprotection studies BDF1 mice were irradiated with 9.5Gy and restored by

transplantation of bone marrow from testosterone-stimulated donors. One group of recipients was restored by bone marrow cells preincubated for 4 hours with medium (controls - group A) and another (group B) with 25 ng/ml INPROL. Cells in both groups were washed and 30,000 cells per mouse were transplanted into irradiated animals. The survival data are shown (Fig. 11). The sum of 3 experiments is depicted, with controls normalized to 100%. INPROL incubation increased the survival of mice from 36.5% in control group up to 61.8% by Day 30.

The increase of MRA induced by preincubation with INPROL could be one of the mechanisms in the improving of the radioprotection. To examine this hypothesis, MRA was measured according to Visser *et al.* (*op. cit.*). Briefly, the donor BDF1 mice were pretreated with testosterone, their bone marrow was preincubated with medium or INPROL for 4 hours and injected into irradiated animals. On Day 13, the bone marrow cells from recipient femurs were plated in agar in 3 different concentration (0.01, 0.05, 0.1 equivalent of a femur) in the presence of 20% of horse serum and 10% of WEHI-CM. The number of Day 7 colonies represented the MRA as far as the colony-forming cells in the bone marrow of recipients at the time were the progenitors of the donor's immature stem cells.

As can be seen on Fig. 12 MRA of preincubated with INPROL cell population is greater than in control group (B).

Example 9: Antihyperproliferative Effect Of INPROL On Stem Cells Can Change Their Differentiation Abnormalities.

Hyperproliferation of CFUs is not only seen during restoration from cytotoxic drugs or irradiation but also as a consequence of normal aging, and is thought to be a major feature in Myelodysplastic Syndrome (MDS). It is accompanied by the differentiation disturbances such as a prevalence of the erythroid differentiation while the differentiation along the granulocytic

Bone marrow cells were incubated for 4 hours at 37°C with 25 ng/ml of INPROL or media (Control), washed and then plated in agar with 20% of horse serum, 2U/ml Erythropoietin, and 10% WEHI-CM. The number of BFU-e and GM-CFU colonies were scored on Day 7. Data presented in Table 5 are summarized from 3 experiments, 4 animals per

point were taken for each group: 4 dishes were plated.

As is obvious from Table 5, the incubation of normal bone marrow (NBM) from intact animals (BDF1 8-12 weeks old) with INPROL did not change the number or proportion of different types of colonies; BDF_1 donors pretreated with Testosterone Propionate (TSP) showed the increase in CFU proliferation as it was seen before (Example 1, 3, 4) and the increase in the erythroid progenitors number (BFUe colonies) and decrease in GM-CFU, which was completely abrogated by the incubation with INPROL. The abnormally high level of CFUs proliferation was returned to the 10% of CFUs in S-phase of cell cycle as well. CFUs hyperproliferation is known to be a feature of some mice strains highly susceptible to viral leukemia induction like for example Balb/c mice (Table 5) and can also be observed in older animals (Table 5). The same redistribution of committed progenitors seen in TSP treated BDF1 mice is observed in Balb/c and 23-25 month old BDF1, which have in common the abnormally high level of CFUs proliferation. The correction of both the proliferation of CFUs and the differentiation was possible by the incubation with INPROL. What is even more clinically relevant, the study showed that the *in vivo* injection of INPROL (2 ug/mouse) would bring the effect both on proliferation of CFUs and change the ratio of erythroid (BFUe) and GM-colonies (Table 5).

Table 5

INPROL Effect On CFUs Differentiation Into Committed Progenitors BFUe and CFU-GM

Donors Of Bone Marrow	INPROL	Percent CFUs Killed by ^{3}H-TdR	BFUe	CFU-GM
BDF_1 Young	-	12.0 ± 0.3	28.33 ± 1.91	46.22 ± 3.44
	+	15.0 ± 1.3	22.00 ± 3.74	47.70 ± 3.72
BDF_1 Old	-	47.1 ± 1.9	43.75 ± 1.54	24.0 ± 1.33
	+	11.4 ± 0.7	15.25 ± 1.45	44.0 ± 7.63
BDF Stimulated by TSP	-	53.2 ± 1.6	32.67 ± 2.44	15.71 ± 2.28
	+	7.2 ± 0.4	12.00 ± 1.83	35.50 ± 1.4
Balb/C	-	57.0 ± 1.9	47.60 ± 2.96	33.57 ± 3.45
	+	23.0 ± 2.4	24.86 ± 2.53	70.60 ± 4.96

Example 10: Immunostimulatory Activity of INPROL

It has been observed that the incubation of the bone marrow cells containing a high proportion of proliferating CFUs with INPROL not only change the cycling of CFUs, but also their differentiation, switching the predominantly erythroid differentiation in favor of granulocytic and lymphoid progenitors. This property of INPROL is of importance due to immunosuppression side effect of cytotoxic chemotherapy, radiotherapy as well as immunosuppression accompanying hyperproliferative stem cell disorders and aging.

The example shows the direct effect of INPROL on differentiation of immature precursors from the Lymphoid Long Term Culture (LLTC) established according to Wittlock & Witte (Ann. Rev. Immun. 3:213-35, 1985) into pre-B progenitors, measured by the formation of colonies in methylcellulose containing IL-7.

LLTC were established as described and fed with fresh LLTC-media (Terry Fox Labs., Vancouver, Canada) twice a week. Nonadherent cells were harvested once a week, washed free of factors and incubated for 4 hours with 25 ng/ml INPROL or medium alone for control. After the incubation, the cells were washed and plated at a concentration of 10^5 cells/ml in methylcellulose, containing 30% FCS, and 10 ng/ml of IL-7. Data from 3 weeks are shown in Figure 13. The number of large pre-B colonies varied in control, increasing with time, but preincubation with INPROL always stimulated the growth of colonies 4 to 8 fold above the control level. This demonstrates an immunostimulatory property of INPROL which is of use in correcting immunodeficient states and increasing desired immune responses, e.g., to vaccination.

Example 11: INPROL Improves Repopulating Ability of Stem Cells -- Long Term Culture Initiating Cells from Patient with CML

Chronic myeloid leukemia (CML) is a lethal malignant disorder of the hematopoietic stem cell. Treatment of CML in the chronic phase with single-agent chemotherapy, combination chemotherapy, splenectomy, or splenic irradiation may control clinical signs and

symptoms, but does not significantly prolong survival. As CML progress from chronic to accelerated stage, standard therapy is not effective. At present, bone marrow transplantation (BMT) is the only known curative therapy for CML. Therapy with unrelated donor BMT is difficult due to histoincompatibility problems. Fewer than 40% of otherwise eligible CML patients will have a suitably matched related donor; therefore autologous transplantation is preferred. *Ex vivo* conditioning of autologous BMT for infusion together with the ability to select nonleukemic (Ph-negative) myeloid progenitors from Ph-positive patients growing in Long Term Culture (LTC) suggest the potential of autologous sources of normal stem cells to allow aggressive and effective therapy of CML.

In the context of BMT, a hematopoietic stem cell may be defined as one having the ability to generate mature blood cells for extensive periods. We have used the human LTC system developed by C. Eaves & A. Eaves both for quantitating stem cell numbers and as a means to manipulate them for therapeutic use. This involves seeding cells onto a pre-established, irradiated human marrow adherent layer, these cultures are then maintained for 5 weeks. The end point is the total clonogenic cell content (adherent + non-adherent) of the cultures harvested at the end of this time. Clonogenic cell output under these conditions is linearly related to the number of progenitors (Long Term Culture Initiating Cells (LTC-IC)) initially added; the average output from individual human LTC-IC is 4 clonogenic progenitors per LTC-IC. It has been shown previously that when marrow from patients with CML is placed under similar conditions, leukemic (Ph-positive) clonogenic cells rapidly decline, by using quantitation of residual normal LTC-IC in patient with CML is possible to select those likely to benefit from intensive therapy supported by transplantation of cultured autograft (Phillips *et al.*, Bone Marrow Transplantation 8:477-487, 1991).

The following procedure was used to examine the effect of INPROL on the number of clonogenic cells (LTC-IC) among bone marrow transplant cells established from the peripheral blood of a patient with CML.

Cultures were initiated as long term cultures on preirradiated stroma. The peripheral blood of a healthy donor was used as the control. CML patient's peripheral blood cells from a

CML patient was preincubated with or without INPROL (25 ng/ml) for 4 hours, washed and placed in LTC-IC system for 10 days and parallel in LTC-IC for 5 weeks. The number of LTC-IC was estimated by the number of clonogenic progenitors by plating both the adherent and non-adherent cells in methylcellulose with the appropriate growth factors (Terry Fox Laboratories, Vancouver, Canada). The mixture of adherent and non-adherent cells from cultures growing for 10 days was preincubated with or without INPROL and placed on preestablished feeders for an additional 8 weeks, harvests were made at Week 4 and 8 for the measurement of the number of clonogenic cells. Data presented on Figure 14 show that there was no loss in LTC-IC during the first 10 days of culture initiated from the healthy donor's bone marrow and approximately 30% of the number of input LTC-IC were still present after 5 weeks in culture. The number of the CML patient's LTC-IC was drastically reduced to about 8% during the 10 day period and did not recover during further incubation, while the preincubation of cells with INPROL increased the LTC-IC level to 30% of initial number and it was maintained during 8 weeks.

Clinically relevant applications of INPROL predicted by these preliminary data include their use in strategies for selectively improving the normal stem cell content of fresh or cultured marrow transplants, strategies for enhancing the recruitment of residual normal stem cells *in vivo* also protocols for transferring new genetic material into human marrow stem cells for the further transplantation into patients.

Example 12: A Method of Isolation of Immunoactive Inhibitor of Proliferation of Stem Blood Cells From Bone Marrow Preparations

The bone marrow was isolated from pigs' ribs. The ribs from the pigs' carcasses were separated and cleaned from the muscle fibers and fat, cut into pieces and the bone marrow was extracted by a hydropress manufactured by the Biophyzpribor. The bone marrow cells are separated by centrifugation in a centrifuge K-70 at 2,000 rpm for 20 minutes. The extract supernatant is then subjected to ultrafiltration through Amicon USA membranes XM-100, PM-30, PM-50, respectively. According to the analysis by electrophoresis, the main component of the product is albumin (see Fig. 1).

Biochemical Purification

The bone marrow extract and protein components of the fractions were analyzed at every step of purification by gel electrophoresis in 10% polyacrylamide, containing 0.1% sodium dodecyl sulfate.

The electrophoresis was conducted at 20Y cm of the gel for five hours. Then the gel was stained in 0.25. Coomassie CBBC250 in a mixture of ethanol:water:acetic acid 5:5:1 for one hour at 20°C and washed in several changes of 7% acetic acid. Up to 7% of sodium dodecyl sulfate and up to 0.5-1% of mercaptoethanol were added to the samples and the samples were incubated for 5 minutes at 70°C prior to loading on the gel.

The activity of the product was evaluated by the method of inhibition of proliferation of stem hematopoietic cells (CFUs). The method is detailed hereafter.

Stage 1. Purification by precipitation with ammonium sulfate.

The activity was purified by precipitation with ammonium sulfate at 25% with saturation of 40 to 80% which was selected based on the results in Table 1.

Table 1

Saturation(%)	0-40	40-60	60-80	80-100
Activity (%)	~37.2-35.4) =1.8%	~37.2-1.8) =35.4%	~37.2-12.8) =24.4%	~37.2-26.1) =11.1%

The amount of the preparation used for testing after each step of purification was determined in accordance with the level of purification and equivalent to the dose of 2 x 10^{-2} mg of the initial product. Activity was determined by the formula:

% Change = %Sa -%Sb

where a is %S in control

b is %S after incubation with the test fraction.

The fraction was desalted in order to lower the concentration of ammonium sulfate 20 times before each testing of activity and before each following purification step.

Stage 2. The impure inhibitor from Stage 1 is applied after desalting and fractionated utilizing ion exchange chromatography in our case DEAE 23 cellulose and then eluted with a gradient of sodium acetate buffer (pH 6.0).

The active fractions of inhibitor elute between 3-5 mM.

The volume of the column was 1 ml and speed of elution was 4 ml hour. The detection was conducted by the chromatograph Millicrome at 230 and 280 nm. Fraction 1 (see Fig. 2) which exhibited the highest activity was isolated and eluted in 5 mM sodium acetate buffer (see Table 2).

Table 2

Fractions	1	2	3	4	5
Activity	46.3-0 =46.3%	46.3-14.1 =32.2%	46.3-42.1 =4.2%	46.3-19.6 =26.7%	46.3-45.1 =1.2%

The electrophoresis data indicates that the main protein contaminant - albumin (see Fig. 3) is removed from this fraction which leads to an additional fourfold purification.

Stage 3. The partially purified inhibitor from Stage 2 is applied directly to a G-75 Sephadex column.

The volume of the column is 20 ml (20 X 1), elution rate is 2 ml/hour. Elution buffer is 50 mM NaCl, 10 mM Tris-HCl, pH 7.5. Detection was conducted on a chromatograph Millichrome at 230 and 280 nm. Fraction 5 which had the highest activity was isolated.

Table 3

Fractions	1	2	3	4	5
Activity	42.2-19.1 =23.1%	42.2-35.2 =7.0%	42.2-21.5 =20.7%	42.2-38.8 =3.4%	42.2-0 =42.2%

Stage 4. Reverse-phase chromatography (Pharmacia FPLC System) utilizing Pro-REC columns was performed on the Ultrasfera matrix 10 ug in 0.1% trifluoracetic acid in an acetonitrile gradient.

The homogeneity of a product with MW 16-17kD is equal to 90% as was shown in analyzing the acrylamide gel with sodium dodecyl sulfate (see Fig. 6). The result is represented in Fig. 4. Activity is determined on fraction 5. The final yield of the product is 5%. As a result, the total amount of protein with MW 16 kD after the purification is 650 ng/ml of the initial product. During the purification process the product was submitted to heat incubation at 70°C for several minutes but no loss of biological activity was detected.

2. The *In Vivo* Stem Cell Proliferation Assay (CFUs).

Immature hematopoietic progenitors -- Colony Forming Units in spleen -- CFUs can be

detected *in vivo* by forming macroscopic colonies in the spleens of lethally irradiated mice, 8-12 days after the intravenous injection of hematopoietic cells (Till & McCulloch, 1961).

The standard CFUs proliferation assay the method of ^{3}H-Thymidine "suicide" is usually applied (Becker *et al.*, 1970). The method is based on incorporation of the radiolabelled Thymidine (^{3}H-Thymidine), the precursor of DNA, into the cells during DNA synthesis (S-phase of cell cycle). The CFUs which are in S-phase of cycle at a time of testing, are to be killed by the high radioactivity and not readily able to form colonies in spleen. Thus, the difference between the number of CFUs formed by the injection of the cell sample incubated without ^{3}H-thymidine and the same cells incubated with ^{3}H-thymidine would show the percentage of the proliferating CFUs.

The inhibitor testing can not be done with the bone marrow stem cell population from unstimulated animals insofar as the inhibitor does effect on cycling CFUs, which are as low as 7-10% in the bone marrow of normal mice.

To stimulate CFUs proliferation phenylhydrazine (PHZ) stimulation, or sublethal irradiation were used (Lord, Br. J. Haem. 34:441-445, 1976).

We have invented the injection of testosterone-propionate (TSP) based on its stimulatory effect on CFUs cycling (Byron *et al.*, Nature 228:1204, 1970) which simplified the testing and did not course any side effect. The TSP induced stimulation of CFUs proliferation within 20-24 hours after injection and the effect could be seen at least 7 days.

The procedure used for the screening of the fractions during purification on the Inhibitor was as follows:

Mice: BDF_1 or CBF_1 mice strains were used throughout all testing.

Donor mice were treated with TSP in dose 10 mg/100 g by intraperitoneal injection of 0.2 ml/mouse in order to induce 30-50% CFUs in S-phase.

Twenty-four hours later the bone marrow is to be taken from the femurs for the cell suspension preparation. Five to ten millions cells per ml are then incubated with different control and test fractions for 3 to 5 hours at 37°C in water bath, with two tubes for each group (hot and cold now).

After 3 to 5 hours, 3H-Thymidine (1 mCi/ml, specific activity 18-25 Ci/m.mole) is to be added to each hot tube in volume 200 µl per 1 ml of cell suspension, nothing added to the cold tubes, continue incubation 30 more minutes at 37°C.

After 30 minutes incubation the kill reaction is to be terminated by adding 10 ml of cold (temperature 4°C) medium containing 400 µg/ml nonradioactive thymidine. Cells are washed off extensively (3 times).

Cells are to be resuspend and diluted to a desirable concentration for the injections (usually 2 - 4 x 10^4 cells per mouse in 0.3-0.5 ml).

Recipient mice 8-10 per group are to be irradiated not later than 6 hours before the injections.

Harvest recipients spleens on day 9-12 and fix in Tellesnitsky's solution, score colonies by eye. Calculate the percentage of cells in S-phase using the formula:

$$\% S = \frac{a - b}{a} 100\%$$

where a - CFUs number without 3H-thymidine

where b - CFUs number with 3H-thymidine

Example 12B: Alternative method for isolating larger quantities of INPROL

Initial isolation

Ribs from fresh pig carcasses are cleaned of muscle fibers and fat, then cut to pieces and soaked in phosphate-buffered saline in the ratio 1:1 (weight/volume). The obtained mixture is crushed by hydraulic press to separate bone marrow from solid bone material.

The suspension of bone marrow cells is collected and filtered of solid particles through four layers of the cheese-cloth. The filtrate is incubated at 56°C for 40 minutes, then cooled in ice-bath to 4°C. The separated precipitate is removed by centrifugation at 10,000 g for 30 minutes at 4°C and discarded.

The clarified supernatant is added dropwise during 30 minutes to 10 volumes of stirred ice-cold acetone containing 1% by volume of concentrated hydrochloric acid. The resulting mixture is kept at 40C for 16 hours for complete formation of the precipitate. Then the

precipitate is pelleted by centrifugation at 20,000 g for 30 minutes at 4°C. This pellet is washed with cold acetone and dried.

HPLC Purification

The pellet is dissolved in HPLC eluent buffer A containing 5% acetonitrile (MeCN) and 0.1% triflouroacetic acid (TFA) to final protein concentration 8-10 mg/ml. This solution (0.5-0.6 ml) is loaded onto 250 x 4.6 mm HPLC column packed with Polisil ODS-300 (10 mcm) and equilibrated with the same buffer A.

The elution is accomplished by gradient of buffer B (90% MeCN, 0.1% TFA) in buffer A at the flow rate of 1 ml/min according to the following program:

Time, min	% of B
0	0
4	0
5	25
25	90

An additional step of 100% B for 5 minutes is used to wash the column prior to reequilibration. Then the column is equilibrated again for returning it to the initial state, and the next portion of the protein solution may be loaded.

During the separation the column effluent is monitored at 280 nm for the detection of protein peaks. Fractions containing the protein material are collected, separated peaks are pooled and rotary evaporated at 30°C to dryness. The obtained residues are dissolved in distilled water and assayed by bioactivity test and by SDS-PAGE (14% gel, reducing conditions). The peak containing the active material is eluted between 70 and 80% of the buffer B and contains the main protein band of 16 kD and the traces of faster moving proteins as assayed by SDS-PAGE.

An analysis of the active material is shown in Figure 15. 500 ug of purified INPROL was loaded onto a C4 reverse phase column (Vydac) and eluted using a linear gradient of 5-

95% acetonitrile in 0.1% trifluroacetic acid. The material eluted as a single peak at 53% acetonitrile (Fig. 15A). When 250 ug of MIP-1 alpha (R&D Systems), however, was run under identical conditions, it eluted at 43.9% acetonitrile (note that earlier peaks prior to 14% acetonitrile are artifactual and due to air bubbles in the detector). Thus, INPROL is substantially more hydrophobic than MIP-1 alpha under these conditions. TGF-beta is known to elute at lower concentrations than that observed for INPROL under these conditions (Miyazono *et al.* J. Biol. Chem. 263:6407-15, 1988).

A gel of the eluted INPROL material is shown in Figure 15C. Lane 1 is the crude material. Lane 2 is molecular weight markers and Lane 3 is the purified material. There are two major bands, one at approximately 14 kD and one at approximately 35 kD. It is believed that the 35 kD band is a multimeric form of the 14 kD band.

WE CLAIM:

1. An inhibitor of stem cell proliferation characterized by the following properties:

(a) specific activity (IC50) ≤ 20 ng/ml in a murine CFU-S assay;

(b) molecular weight is greater than 10,000 daltons and less than 100,000 daltons by ultra filtration; and

(c) more hydrophobic than either MIP1α or TGFβ by reverse phase chromatography.

2. An inhibitor of stem cell proliferation according to claim 1 wherein said inhibitor is a protein.

3. An inhibitor of stem cell proliferation according to claim 1 wherein said inhibitor is non-inflammatory.

4. An inhibitor of stem cell proliferation according to claim 1 wherein said inhibitor is heat stable.

5. An inhibitor of stem cell proliferation according to claim 1 wherein said inhibitor is acid stable.

6. An inhibitor of stem cell proliferation according to claim 1 wherein said inhibitor has activity in gastrointestinal cells, gastrointestinal tissue, epithelial cells, epithelial tissue, reproductive cells, or reproductive tissue.

7. An inhibitor of stem cell proliferation according to claim 1 wherein said inhibitor has activity in hematopoietic cells or tissue.

8. An inhibitor of stem cell proliferation according to claim 1 wherein said specific activity is ≤ 2ng/ml in a murine CFU-S assay.

9. A pharmaceutical composition comprising an inhibitor according to claim 1 together with a pharmaceutically acceptable carrier or diluent.

10. A pharmaceutical composition according to claim 9 for parenteral administration.

11. An inhibitor of stem cell proliferation having suppressive activity which does not require constant presence of the factor in an *in vitro* proliferation assay.

12. An inhibitor of stem cell proliferation having suppressive activity according to claim 11 wherein said inhibitor is a protein.

13. An inhibitor of stem cell proliferation according to claim 11 wherein said inhibitor is non-inflammatory.

14. An inhibitor of stem cell proliferation according to claim 11 wherein said inhibitor is heat stable.

15. An inhibitor of stem cell proliferation according to claim 11 wherein said inhibitor is acid stable.

16. An inhibitor of stem cell proliferation according to claim 11 wherein said inhibitor has activity in gastrointestinal cells, gastrointestinal tissue, epithelial cells, epithelial tissue, reproductive cells, or reproductive tissue.

17. An inhibitor of stem cell proliferation according to claim 11 wherein said inhibitor has activity in hematopoietic cells or tissue.

18. An inhibitor of stem cell proliferation according to claim 11 wherein said specific activity is $\leq$ 2ng/ml in a murine CFU-S assay.

19. A pharmaceutical composition comprising an inhibitor according to claim 11 together with a pharmaceutically acceptable carrier or diluent.

20. A composition according to claim 19 for parenteral administration.

21. An inhibitor of stem cell proliferation having suppressive activity which is active when present only during a short pre-incubation period.

22. An inhibitor of stem cell proliferation having suppressive activity according to claim 21 wherein said inhibitor is a protein.

23. An inhibitor of stem cell proliferation according to claim 21 wherein said inhibitor is non-inflammatory.

24. An inhibitor of stem cell proliferation according to claim 21 wherein said inhibitor is heat stable.

25. An inhibitor of stem cell proliferation according to claim 21 wherein said inhibitor is acid stable.

26. An inhibitor of stem cell proliferation according to claim 21 wherein said inhibitor has activity in gastrointestinal cells, gastrointestinal tissue, epithelial cells, epithelial tissue, reproductive cells, or reproductive tissue.

27. An inhibitor of stem cell proliferation according to claim 21 wherein said inhibitor has activity in hematopoietic cells or tissue.

28. An inhibitor of stem cell proliferation according to claim 21 wherein said specific activity is $\leq$ 2ng/ml in a murine CFU-S assay.

29. A pharmaceutical composition comprising an inhibitor according to claim 21 together with a pharmaceutically acceptable carrier or diluent.

30. A composition according to claim 29 for parenteral administration.

31. A method for stimulating the growth of B cells which comprises administering an effective amount of an inhibitor according to claim 1 to promote lymphoid development.

32. A method for treating cancer comprising :

(a) removing bone marrow from a patient;

(b) treating said bone marrow in a culture with an inhibitor:

(c) treating said bone marrow with a cytotoxic chemotherapy agent to destroy the cancer cells; and

(d) transplanting into a patient following myeloablative treatment.

33. A method for treating leukemia comprising :

(a) removing bone marrow from a patient;

(b) treating said bone marrow in a culture with the inhibitor:

(c) treating said bone marrow with a cytotoxic chemotherapy agent to destroy the leukemia cells; and

(d) transplanting into a patient following myeloablative treatment.

34. A pharmaceutical composition useful for inhibiting stem cell division in a mammal exposed to an agent capable of damaging or destroying stem cells undergoing division.

35. The pharmaceutical composition according to claim 34 wherein said inhibitor of stem cell proliferation is used for the successful maintenance or expansion of mammal hematopoietic stem cells *ex vivo* in long term cultures of bone marrow or peripheral blood or cord blood cells for the purposes of bone marrow transplantation (autologous or allogeneic) or for gene transfer.

36. The composition according to claim 34 wherein said stem cell inhibitory factor is used for the treatment of hyperproliferation of hematopoietic stem cells in myeloproliferative or autoimmune diseases or epithelial stem cells hyperproliferation.

37. An inhibitor of stem cell proliferation isolated by the following steps:

(a) isolating bone marrow and removing particulate matter from an extract;

(b) heating said extract and removing precipitate;

(c) acid precipitating said extract and collecting precipitate; and

(d) isolating said inhibitor by reverse phase chromatography.

38. A pharmaceutical composition comprising an inhibitor according to claim 37 together with a pharmaceutically acceptable carrier or diluent.

39. A composition according to claim 38 for parenteral administration.

40. A pharmaceutical composition useful for differentially protecting normal stem cells, and not leukemia cells, from chemotherapy or radiation.

41. A method of treatment for mammals wherein said inhibitor of stem cell proliferation is administered after the stem cells were induced to proliferate by exposure to a cytotoxic drug or irradiation procedure.

42. A method of treatment for mammals wherein said inhibitor of stem cell proliferation is administered as an adjuvant before or together with vaccination based on the ability to increase the immune response.

43. A method for purifying an inhibitor of stem cell proliferation substantially free from other proteinaceous material comprising the following steps:

(a) isolating bone marrow and removing particulate matter from an extract;

(b) heating said extract and removing precipitate;

(c) acid precipitating said extract and collecting precipitate; and

(d) isolating said inhibitor by reverse phase chromatography.

44. A method of treatment for mammals which comprises administration of an inhibitor of stem cell proliferation according to claim 1 so as to protect hematopoietic stem cells against damage by cytotoxic drugs.

45. A method of treatment for mammals which comprises administration of an inhibitor of stem cell proliferation according to claim 11 so as to protect hematopoietic stem cells against damage by cytotoxic drugs.

46. A method of treatment for mammals which comprises administration of an inhibitor of stem cell proliferation according to claim 21 so as to protect hematopoietic stem cells against damage by cytotoxic drugs.

47. A method of treatment for mammals which comprises administration of an inhibitor of stem cell proliferation according to claim 37 so as to protect hematopoietic stem cells against damage by cytotoxic drugs.

48. A method of treatment for mammals wherein an inhibitor of stem cell proliferation functions to reverse immunodepression caused by stem cell hyperproliferation.

49. An antibody to an inhibitor according to claim 1.

50. An antibody to an inhibitor according to claim 11.

51. An antibody to an inhibitor according to claim 21.

52. An antibody to an inhibitor according to claim 37.

53. An inhibitor of stem cell proliferation according to claim 1 used in conjunction with antivirals.

54. An inhibitor of stem cell proliferation according to claim 11 used in conjunction with antivirals.

55. An inhibitor of stem cell proliferation according to claim 21 used in conjunction with antivirals.

56. An inhibitor of stem cell proliferation according to claim 37 used in conjunction with antivirals.

57. A method of treatment of leukemia in mammals which comprises the suppression of leukemia cells which are sensitive to the inhibitor.

58. An inhibitor of stem cell proliferation, immunologically distinct from MIP1α or TGFβ, having a specific activity $\leq$ 2ng/ml in a murine CFU-S assay.

59. An inhibitor of stem cell proliferation according to claim 58 wherein said inhibitor is a protein.

60. An inhibitor of stem cell proliferation according to claim 58 wherein said inhibitor is non-inflammatory.

61. An inhibitor of stem cell proliferation according to claim 58 wherein said inhibitor is heat stable.

62. An inhibitor of stem cell proliferation according to claim 58 wherein said inhibitor is acid stable.

63. An inhibitor of stem cell proliferation according to claim 58 wherein said inhibitor has activity in gastrointestinal cells or tissue, epithelial cells or tissue, or reproductive cells or tissue.

64. An inhibitor of stem cell proliferation according to claim 58 wherein said inhibitor has activity in hematopoietic cells or tissue.

65. A pharmaceutical composition comprising an inhibitor according to claim 58 together with a pharmaceutically acceptable carrier or diluent.

66. A pharmaceutical composition according to claim 65 for parenteral administration.

67. An inhibitor of stem cell proliferation, immunologically distinct from MIP1α or TGFβ, having a specific activity ≤ 5ng/ml in a murine CFU-S assay.

68. An inhibitor of stem cell proliferation, immunologically distinct from MIP1α or TGFβ, having a specific activity ≤ 1ng/ml in a murine CFU-S assay.

69. A gene comprising a recombinant DNA molecule encoding a polypeptide possessing biological activity, immunologically distinct from MIP1α or TGFβ, wherein said biological activity is the inhibition of stem cell proliferation.

70. An analogue to the inhibitor according to claim 1.

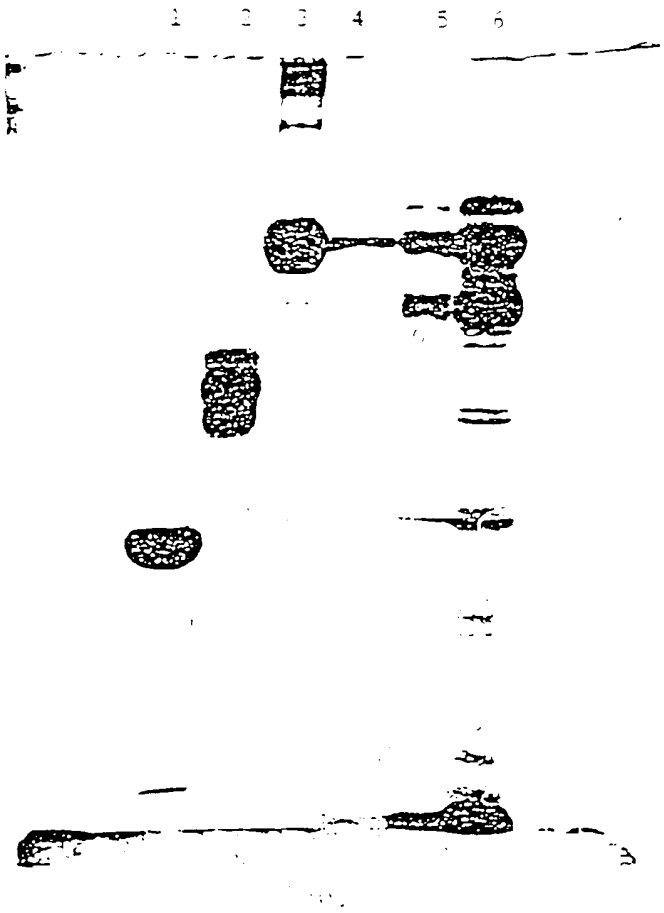


Figure 1

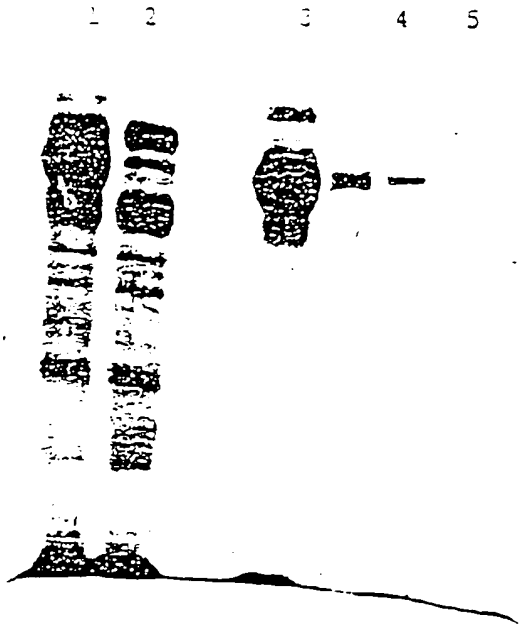


Figure 2

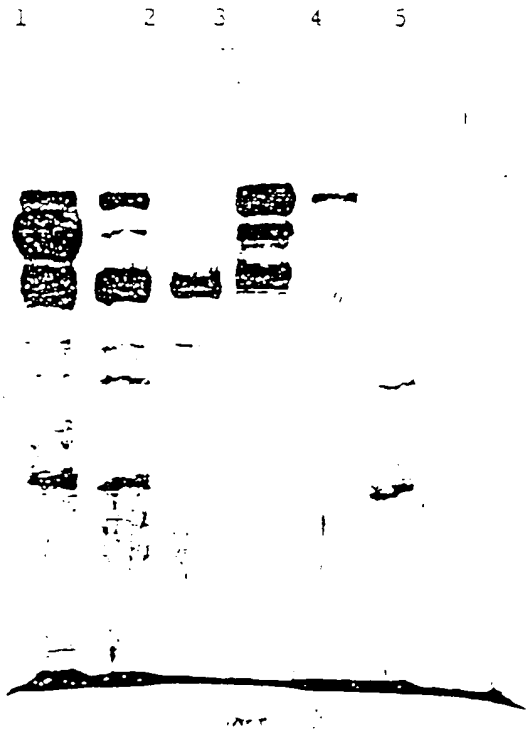


Figure 3

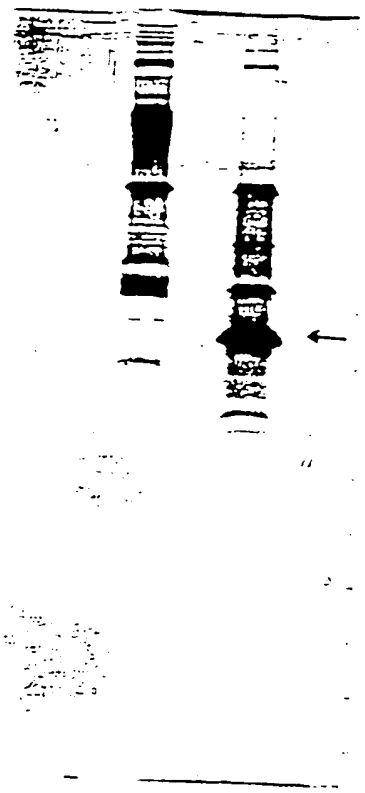


Fig 4

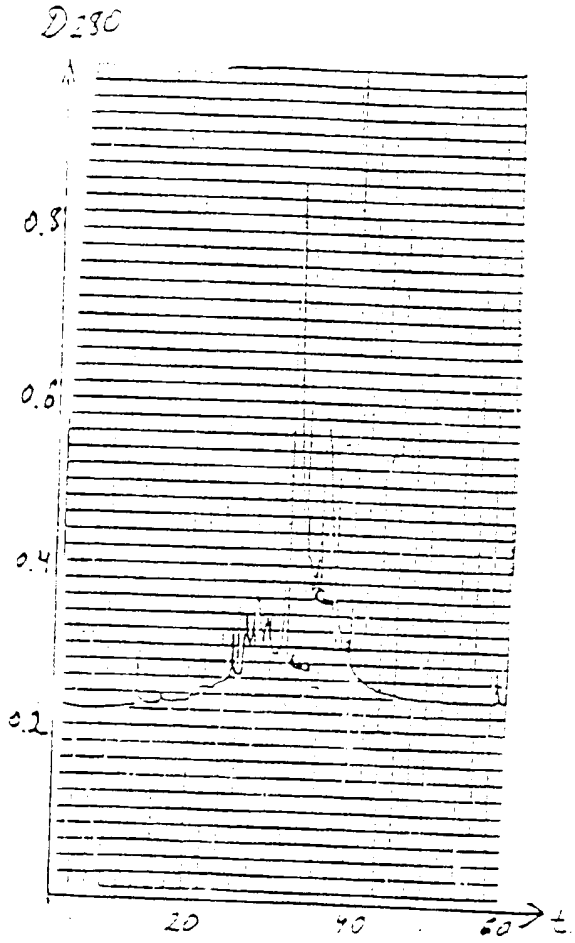


Fig. 5

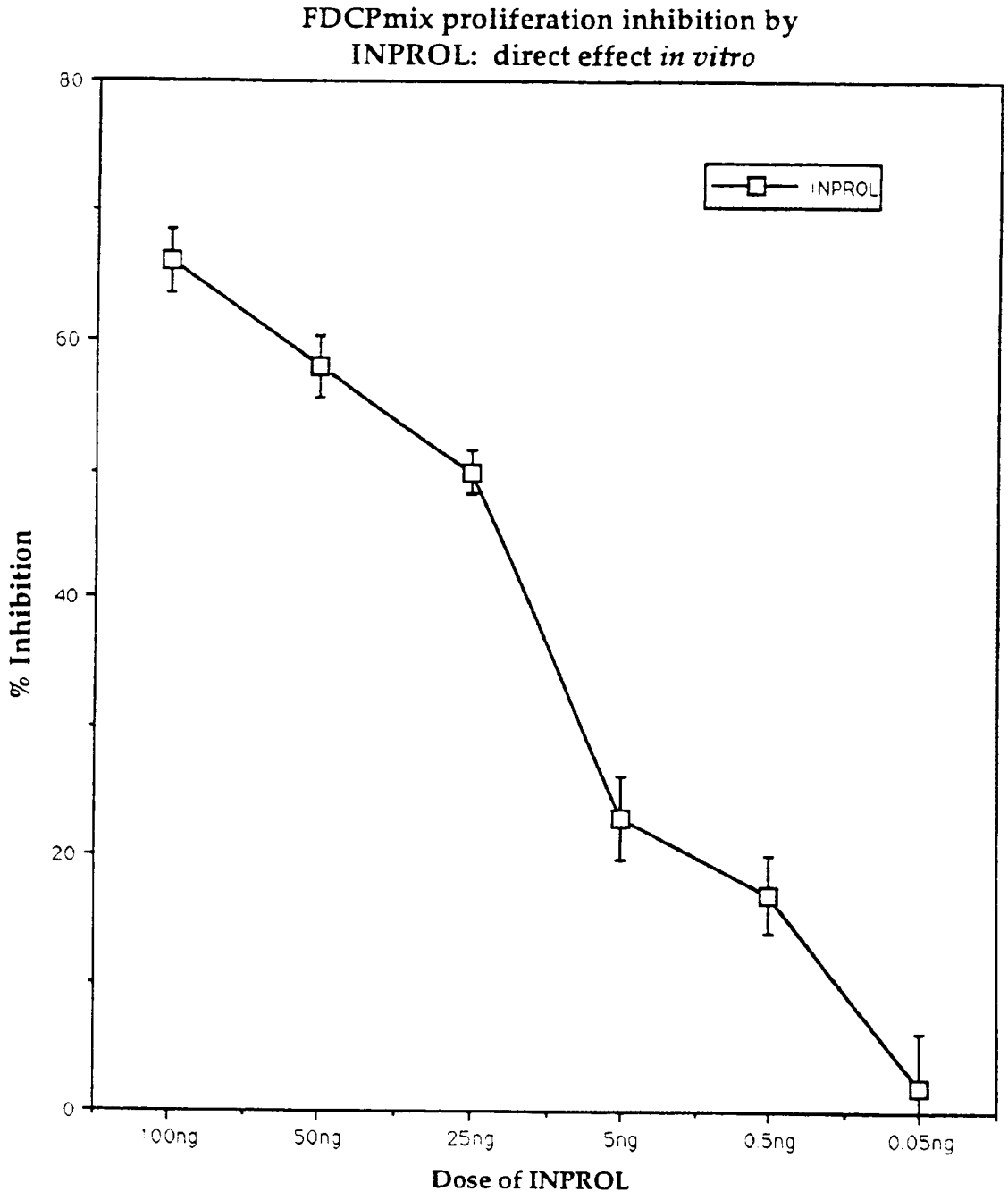

FDCPmix proliferation inhibition by
INPROL: direct effect *in vitro*
80
60
40
20
0
% Inhibition
INPROL
100ng
50ng
25ng
5ng
0.5ng
0.05ng
Dose of INPROL

Fig. 6

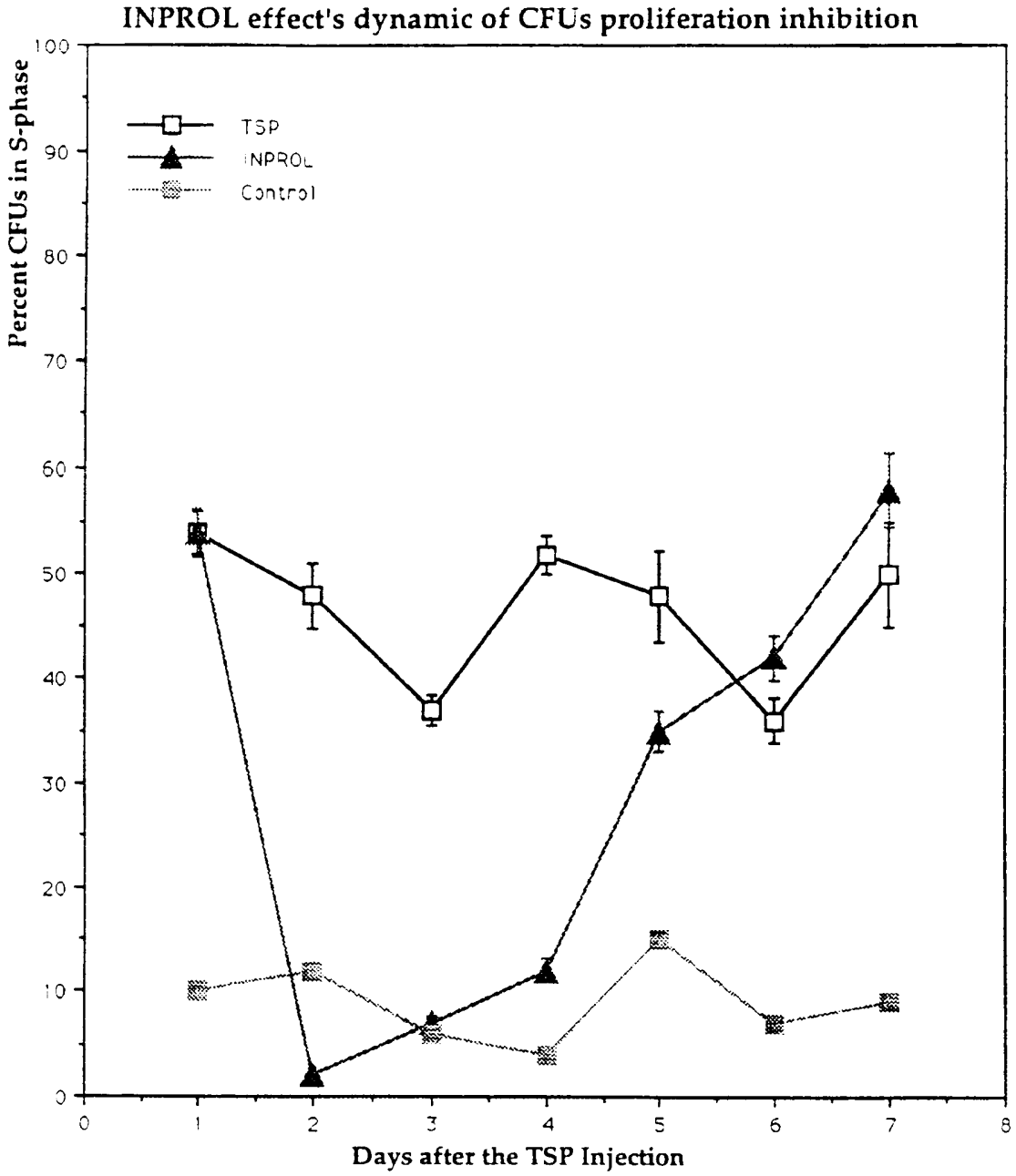

INPROL effect's dynamic of CFUs proliferation inhibition
Percent CFUs in S-phase
100
90
80
70
60
50
40
30
20
10
0
TSP
INPROL
Control
0
1
2
3
4
5
6
7
8
Days after the TSP Injection

Fig. 7

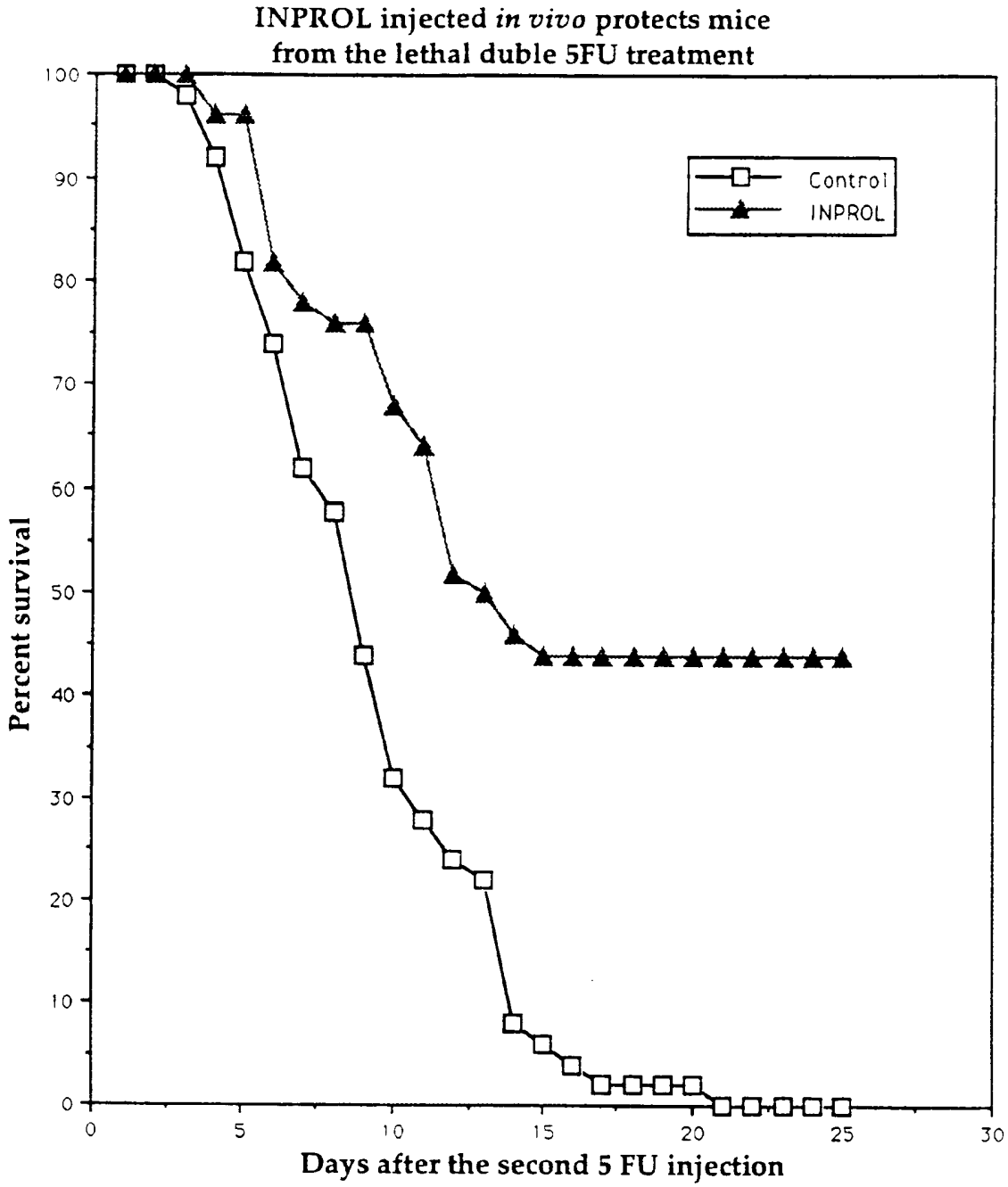

INPROL injected *in vivo* protects mice
from the lethal duble 5FU treatment
Percent survival
Days after the second 5 FU injection
Control
INPROL
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30

Fig. 8

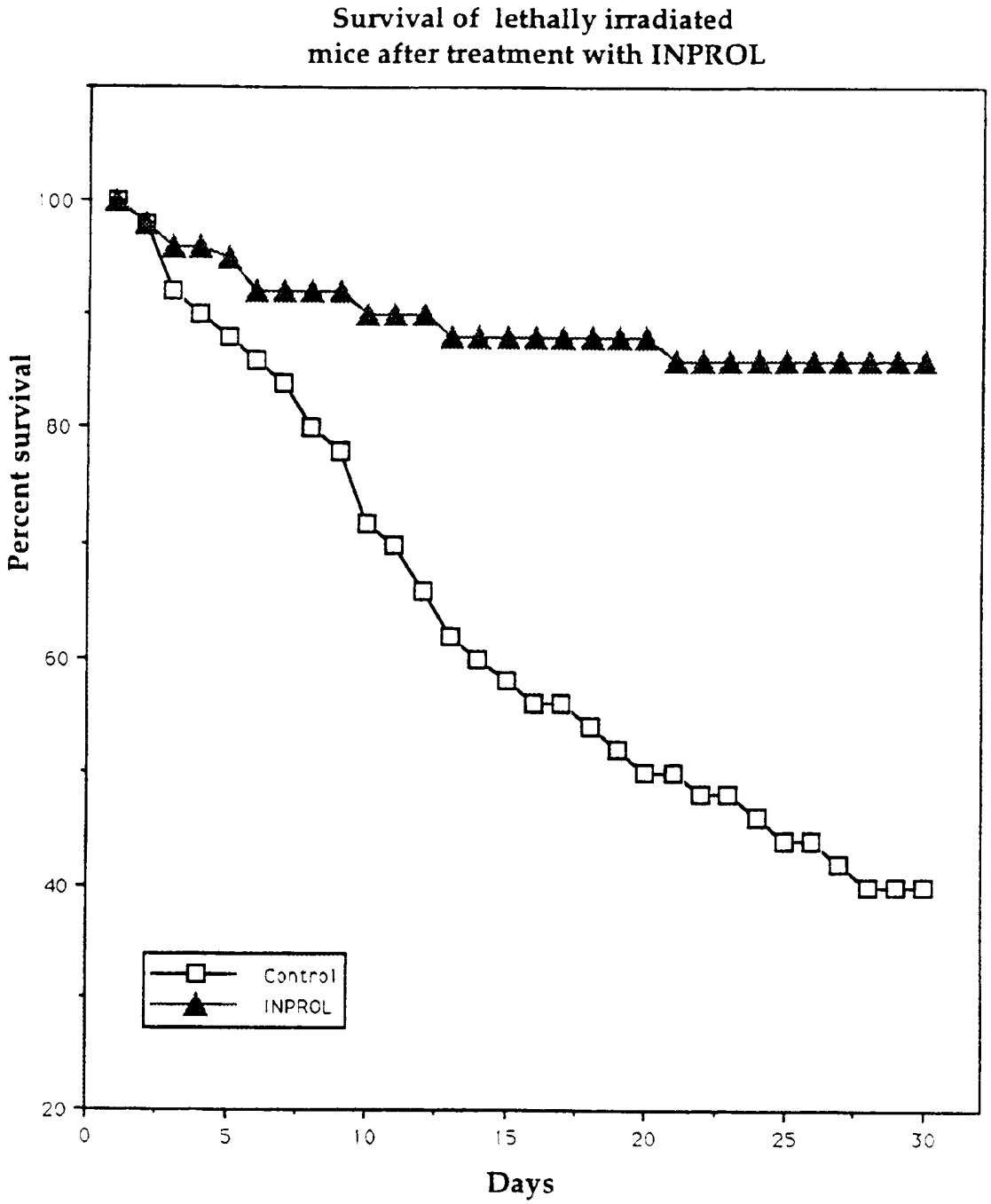

Survival of lethally irradiated
mice after treatment with INPROL
Percent survival
100
80
60
40
20
0
5
10
15
20
25
30
Days
Control
INPROL

Fig. 9

Cell regeneration in BMLTC - L1210 cultures after combined AraC plus Inprol treatment

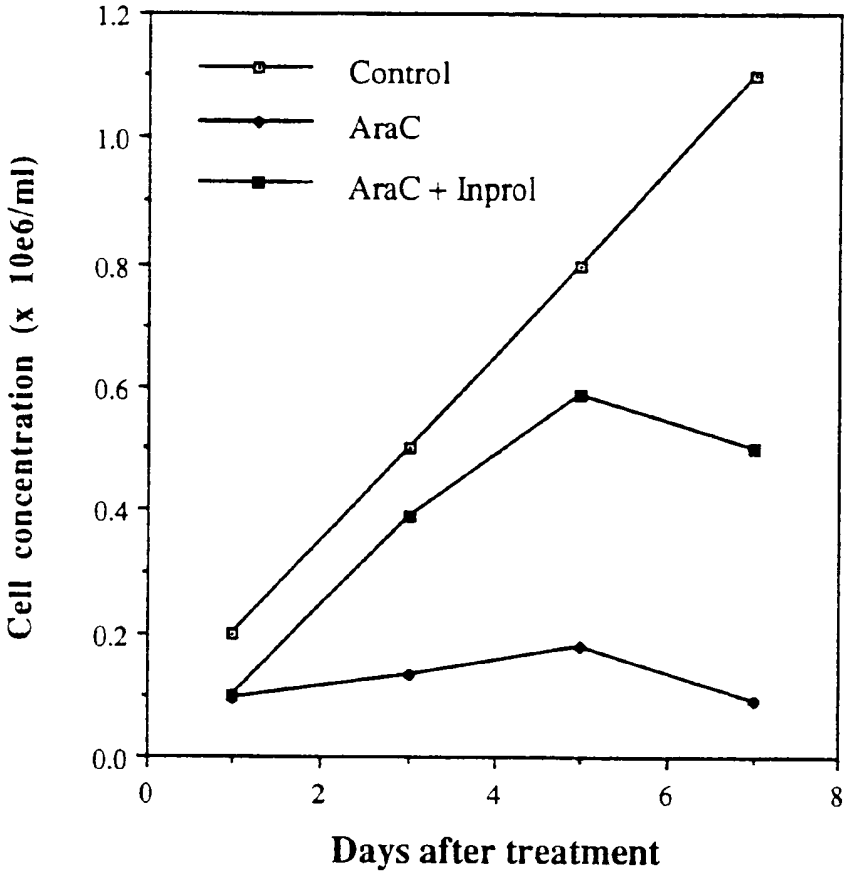


Figure 10 A

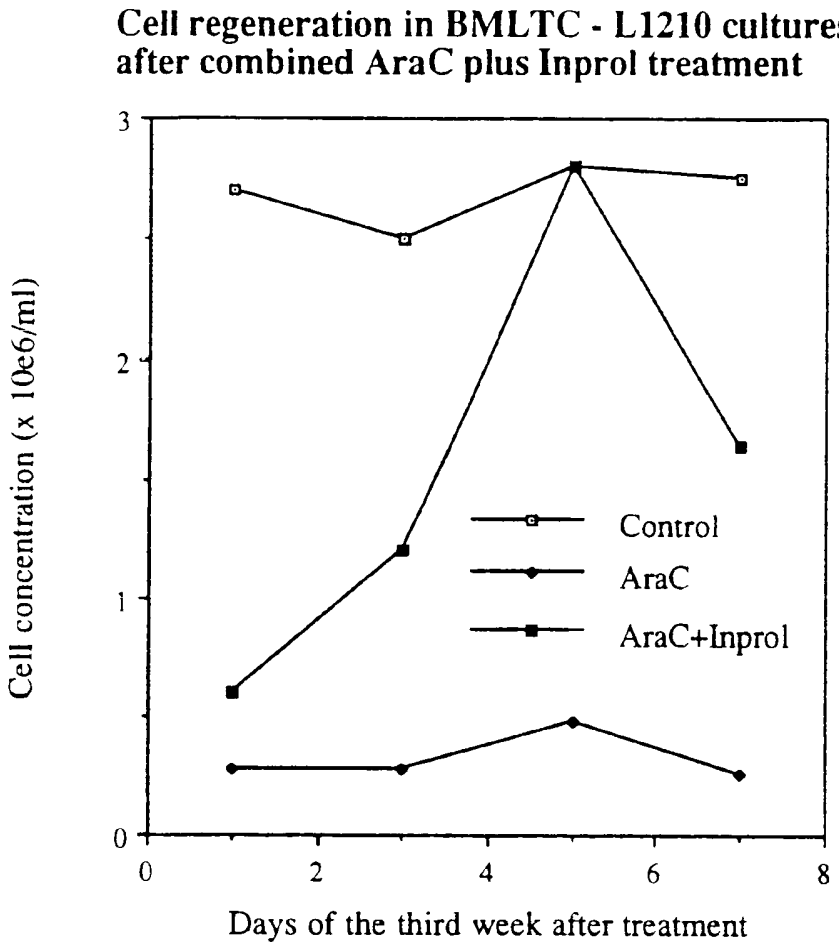

Cell regeneration in BMLTC - L1210 cultures after combined AraC plus Inprol treatment
Cell concentration (x 10e6/ml)
3
2
1
0
0
2
4
6
8
Control
AraC
AraC+Inprol
Days of the third week after treatment

Figure 10B

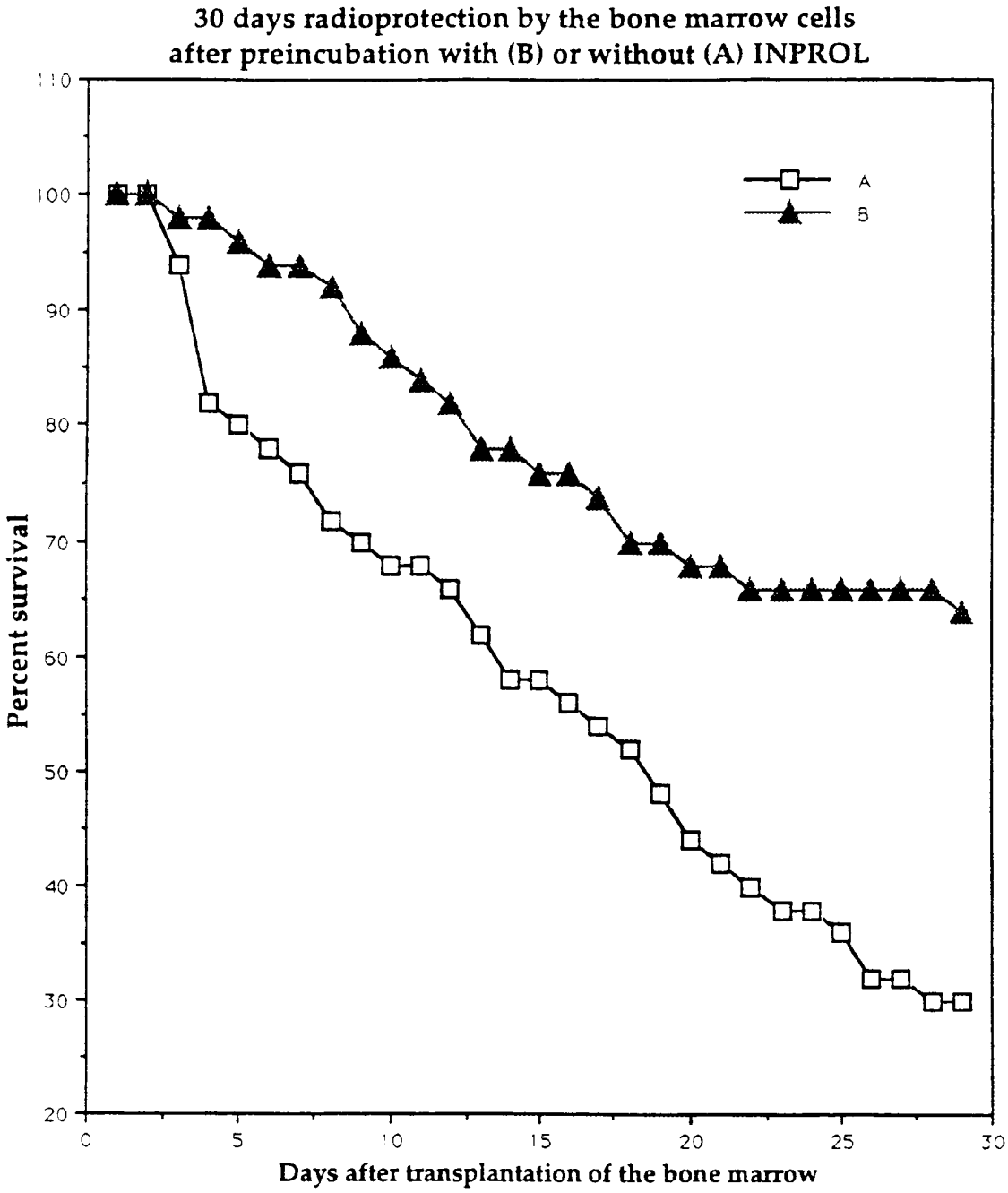

30 days radioprotection by the bone marrow cells
after preincubation with (B) or without (A) INPROL
Percent survival
Days after transplantation of the bone marrow
A
B
110
100
90
80
70
60
50
40
30
20
0
5
10
15
20
25
30

Fig. 11

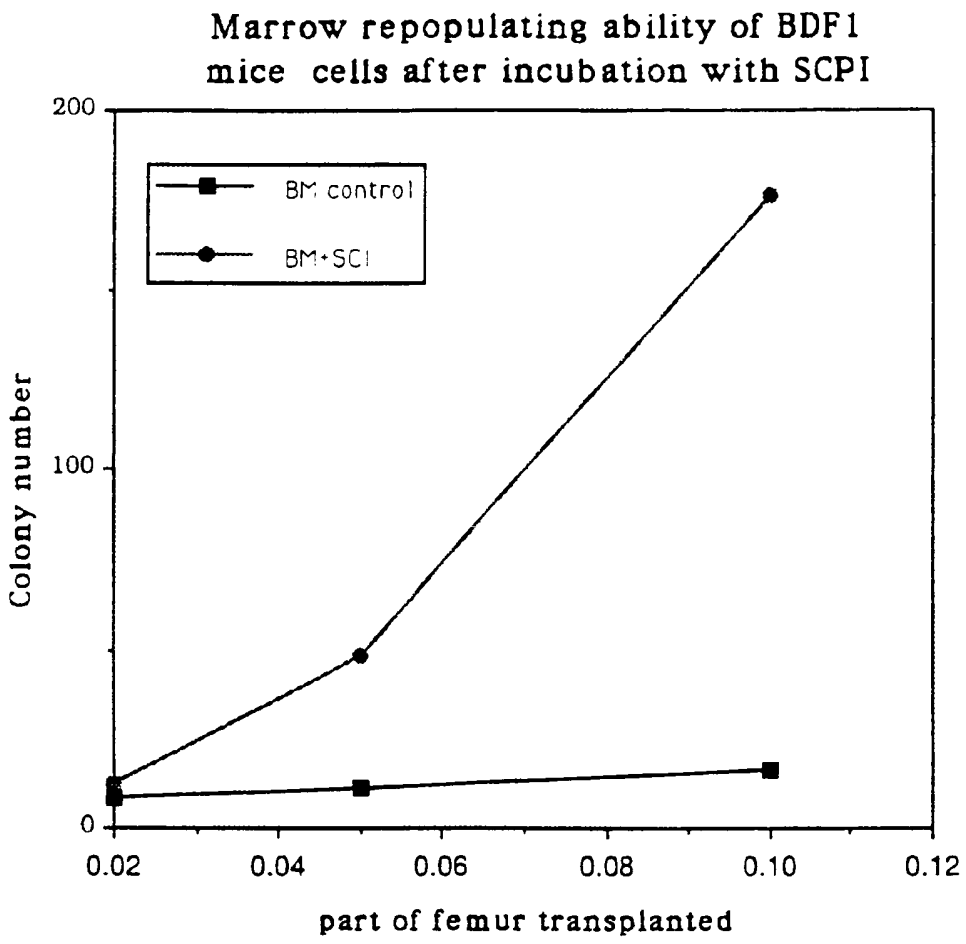

Marrow repopulating ability of BDF1 mice cells after incubation with SCPI
200
100
0
Colony number
BM control
BM+SCI
0.02
0.04
0.06
0.08
0.10
0.12
part of femur transplanted

Fig. 12

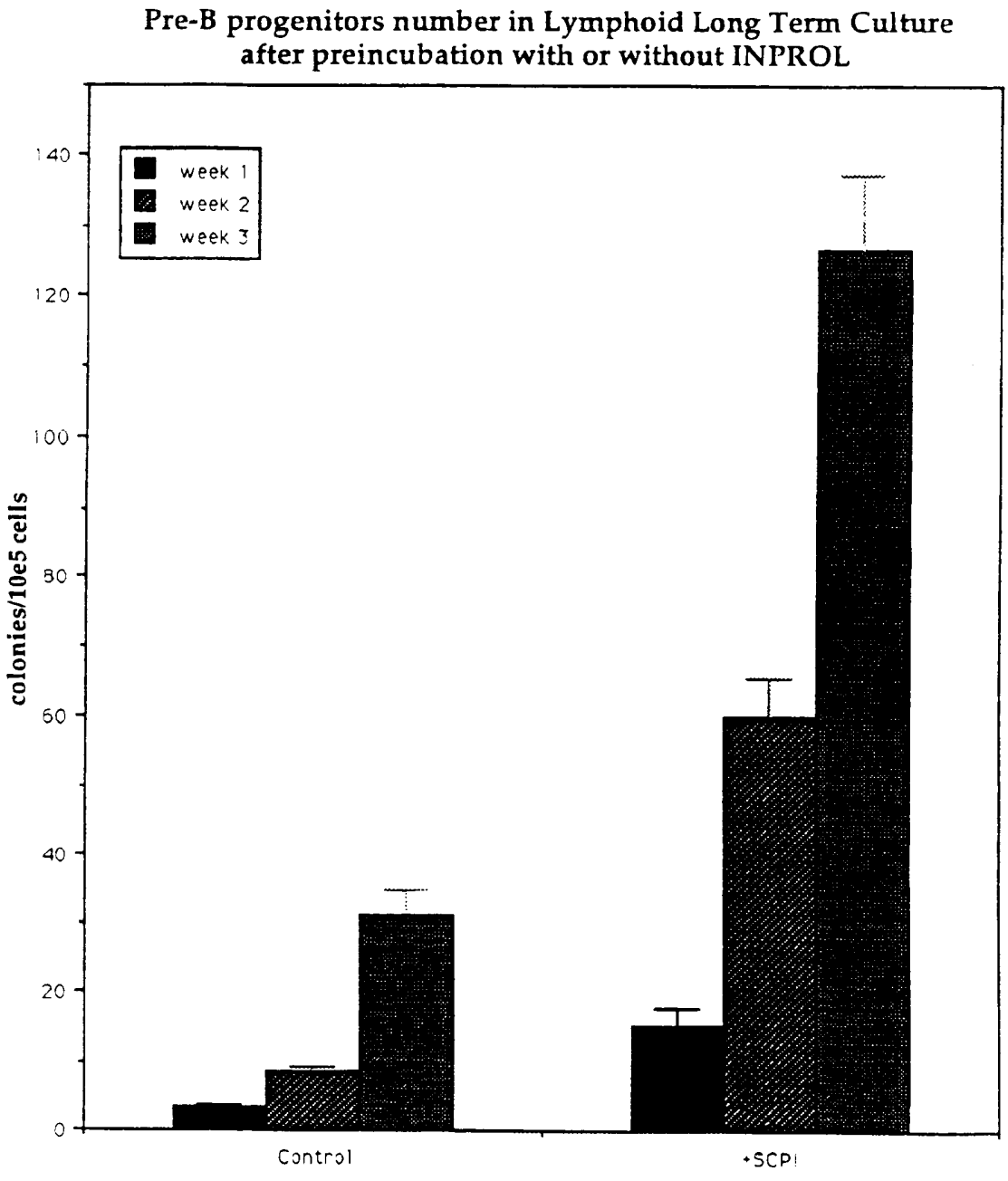

Pre-B progenitors number in Lymphoid Long Term Culture
after preincubation with or without INPROL
week 1
week 2
week 3
colonies/10e5 cells
140
120
100
80
60
40
20
0
Control
+SCPI

Fig. 13

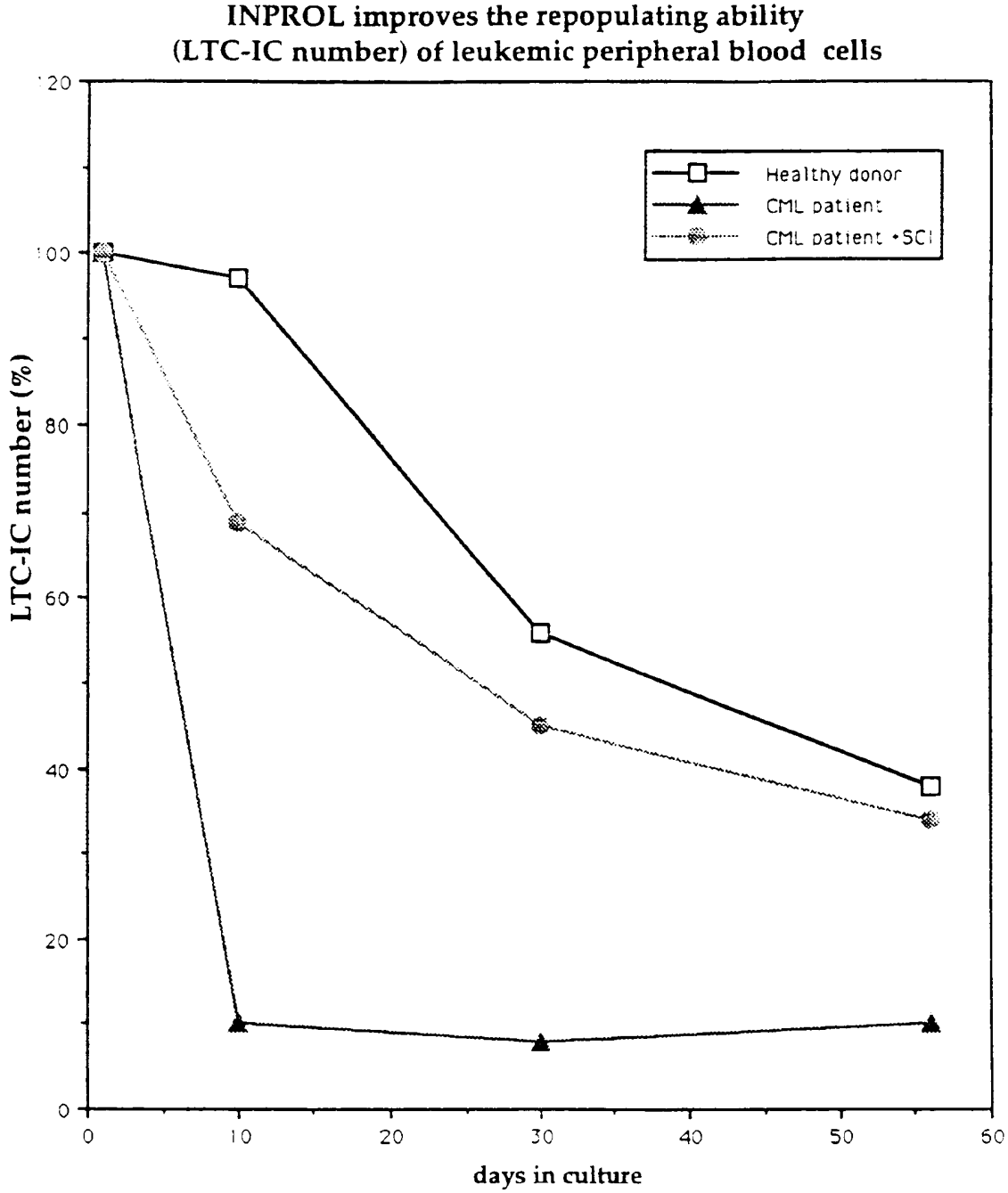
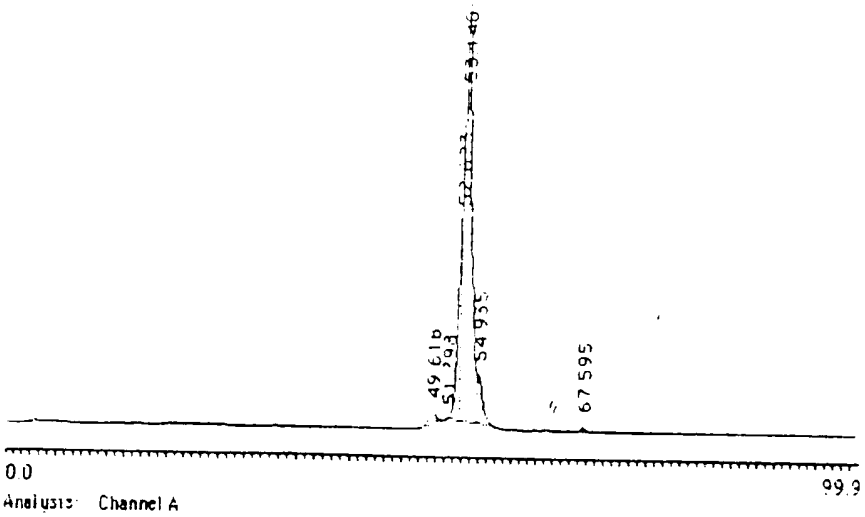

INPROL improves the repopulating ability
(LTC-IC number) of leukemic peripheral blood cells
Healthy donor
CML patient
CML patient +SCF
LTC-IC number (%)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
days in culture

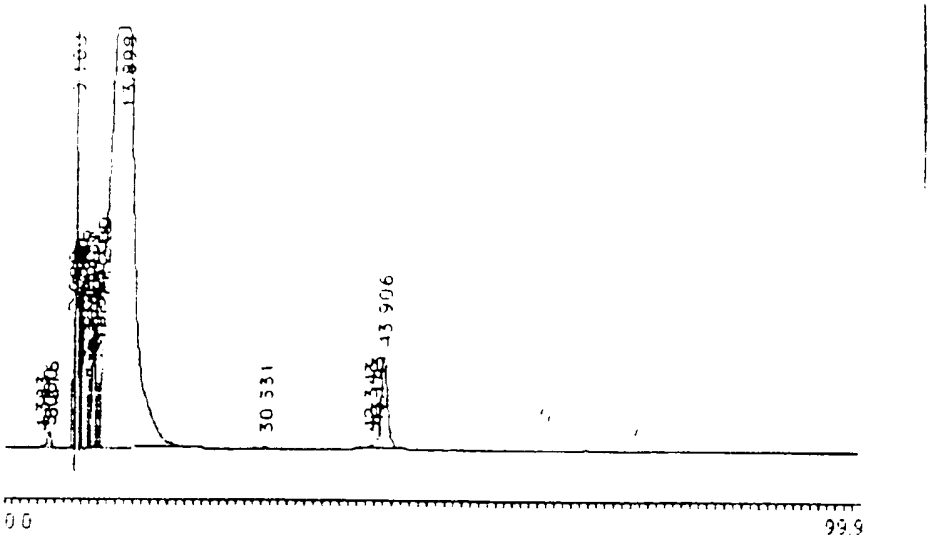
Fig 14



Analysis: Channel A

Peak No	Time	Type	Height(μV)	Area(μV-sec)	Area%
	3.126	N1	691	7578	0.041
	3.315	N2	1011	5150	0.027
1	49.618	N	8584	349227	1.893
2	51.298	N	1456	20274	0.109
3	52.673	N1	138069	2633395	14.278
4	53.148	N2	271587	14050458	76.181
5	54.935	N3	33016	1332820	7.226
6	67.595	N	3270	44507	0.241
Total Area				18443409	99.996

Fig. 15A



Analysis Channel A

Peak No	Time	Type	Height(μY)	Area(μY-sec)	Area%
1	4.383	N1	3945	95125	0.119
2	5.080	N2	28639	330889	0.413
3	5.216	N3	49084	531867	0.665
4	7.980	N1	399424	1110511	1.389
5	8.100	Err	1203320	2882013	3.605
6	8.241	N3	443249	1506159	1.884
7	8.386	N4	481563	2185702	2.734
8	8.533	N5	412886	1826165	2.284
9	8.701	N6	321500	842122	1.053
10	8.745	N7	404661	1610380	2.014
11	8.995	N8	435765	2489721	3.114
12	9.316	N9	517790	4801831	6.007

Fig. 15B

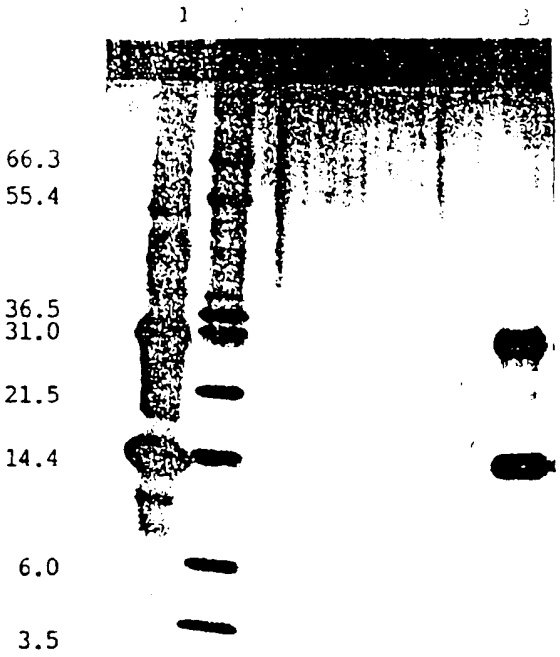


Fig. 15C

INTERNATIONAL SEARCH REPORT

Inte...tional application No.
PCT/US94/03349

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 15/00, 3/28; C07H 21/04; A61K 37/02, 39/00
US CL :530/350, 387.1, 412; 514/2; 424/85.8; 536/23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 387.1, 412; 514/2; 424/85.8; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG/CAS/MEDLINE/APS
search terms: stem cell, hematopoietic stem cell, inhibitor, bone marrow, heat stable, NBME-IV, SCI, INPROL, MIP-1alpha

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell & Tissue Kinetics, Volume 20, issued 1987, Kozlov et al. "The Effect of Haematopoietic Stem Cell Proliferation on the Humoral Immune Response in Mice", pages 485-491, see page 485, Abstract and second paragraph, and Figure 1 on page 487.	1-42, 44-47
Y	Blood, Volume 78, Number 1, issued 01 July 1991, Eaves et al., "Mechanisms That Regulate the Cell Cycle Status of Very Primitive Hematopoietic Cells in Long-Term Human Marrow Cultures. II. Analysis of Positive and Negative Regulators Produced by Stromal Cells Within the Adherent Layer", pages 110-117, see page 114, left hand column, line 5 to right hand column, line 8 and page 115, right had column, lines 1-4.	1-8, 11-18, 21-28, 37, 49-52

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 JULY 1994	Date of mailing of the international search report JUL 13 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer George C. Elliott Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Inte ional application No.
PCT/US94/03349

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	British Journal of Cancer, Volume 50, issued 1984, Tejero et al., "The Cellular Specificity of Haematopoietic Stem Cell Proliferation Regulators", pages 335-341, see page 336, left hand column, first full paragraph, and page 338, paragraph bridging the two columns and the first full paragraph of the right hand column.	1-8, 11-18, 21-28, 37
X --- Y	Nature, Volume 344, issued 29 March 1990, Graham et al., "Identification and Characterization of an Inhibitor of Haematopoietic Stem Cell Proliferation", pages 442-444, see entire document.	41, 58, 59, 61-66 --- 1-40, 44-53, 57-67, 69, 70
X --- Y	WO, A, 91/04274 (PRAGNELL ET AL.) 04 April 1991, see entire document.	41, 58, 59, 61 --- 1-40, 44-53, 57-67, 69, 70